IMPACT OF MILK PRESERVATIVES ON ANTIMICROBIAL RESISTANCE

Project report submitted to Sister Nivedita University in partial fulfillment of the requirement of the degree of

MASTER OF SCIENCE
IN
MICROBIOLOGY

Submitted By

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TO WHOM IT MAY CONCERN

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I hereby declare that the work which is being presented in this dissertation entitled "**Impact of Milk Preservatives on Antimicrobial Resistance**" in partial fulfilment of the requirements for the award of the degree of **Masters of Science in MICROBIOLOGY** is an authentic record of my own work carried out during the period from **February 2025 to May 2025** under the supervision of **Mr. Mayukh Bose Department of BOSE INFORMATICS.**

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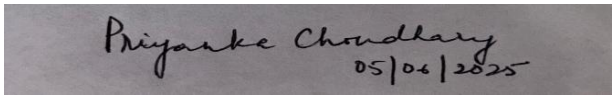

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LIST OF FIGURES:

FIGURE NO.	TITLE	PAGE NO.
Figure 1.	Sample of shed milk	12
Figure 2.	Growth of microorganisms	13
Figure 3.	Pure culture plate 1	14
Figure 4.	Pure culture plate 2	14
Figure 5(a & b)	Catalase test	15
Figure 6.	Indole test	15
Figure 7.	Simmon Citrate test	16
Figure 8.	Methyl Red test	16
Figure 9.	Voges-Proskauer test	17
Figure 10(a &b)	Gram staining	18
Figure 11(a &b)	Carbohydrate test	21
Figure 12.	Graphical representation of carbohydrate	22
Figure 13(a &b)	Sugar test results	25-26
Figure 14(a &b)	Protein test	28
Figure 15.	Graphical representation of protein	29
Figure 16 -20	Fat estimation	31-34
Figure 20-21	Preservative estimation of commercial milk	35-40
Fig 22-23(a &b)	Antimicrobial resistance	45-46
Fig 24-27	Minimum Inhibitory Concentration	50-58

LIST OF TABLES –

SL.NO	TITLE	PAGE NO.
Table 1.	Biochemical identification results of bacterial isolates	20
Table 2.	Absorbance reading of carbohydrate	22
Table 3.	Absorbance reading of protein	29
Table 4.	Preservative data of commercial milk	38
Table 5.	Standard data of potassium sorbate	39
Table 6.	Standard data of natamycin	40
Table 7.	Zone of inhibition	47
Table 8-11	Minimum and maximum dosage of preservatives in *E. coli* and *S. aureus*	51-53
Table 12-15	Minimum and maximum dosage of mixed preservatives in *E. coli* and *S. aureus*	54-56

List of Abbreviations

Abbreviation	Full Form
AMR	Antimicrobial Resistance
MIC	Minimum Inhibitory Concentration
CFU	Colony-Forming Unit
E. coli	*Escherichia coli*
S. aureus	*Staphylococcus aureus*
FSSAI	Food Safety and Standards Authority of India
ISO	International Organization for Standardization

ABSTRACT

Milk is a staple dietary component rich in essential nutrients, but it is often subjected to adulteration and preservation using chemical agents. Both commercial and shed (locally sourced) milk samples were analysed for the presence of these compounds using spectrophotometric techniques. Calibration curves for potassium sorbate and natamycin were plotted. Their absorbance was measured at specific wavelengths (254 nm for potassium sorbate and 318.2, 304, 296 nm for natamycin) and applied to determine preservative concentrations in unknown samples.

Microbial isolates such as *E. coli* and *Staphylococcus aureus* were obtained from the milk samples and tested for antimicrobial susceptibility via the MIC (Minimum Inhibitory Concentration) method. Nutrient agar was used for microbial growth, followed by serial dilution of milk and antimicrobial assays. Biochemical tests were employed to confirm microbial identity, including urease, catalase, and sugar fermentation.

The results revealed potassium sorbate concentration in milk at approximately 48.88 mg/L and natamycin at an average of 39.04 mg/L. These concentrations demonstrated varying effects on microbial growth, indicating resistance in some isolates. The data suggest a potential link between preservative exposure and emerging antimicrobial resistance in foodborne microbes. This raises concerns about long-term public health impacts and regulatory policies concerning food preservatives.

The project highlights the importance of routine monitoring of milk quality and calls for stricter guidelines on the use of preservatives in food industries to mitigate the risk of AMR development.

Keywords –

Antimicrobial resistance (AMR), milk preservatives, potassium sorbate, natamycin, Escherichia coli, Staphylococcus aureus, minimum inhibitory concentration (MIC), food adulterants, spectrophotometric analysis, bacterial isolates, commercial milk, shed milk, food safety, UV-Visible spectrophotometry, microbial tolerance.

CONTENTS –

ACKNOWLEDGEMENT……5

LIST OF FIGURES……...6

LIST OF TABLES…….7

ABBREVIATIONS…….7

ABSTRACT…… 8

INTRODUCTION……...10

LITERATURE REVIEW……11

MATERIALS AND METHODS……13

RESULT AND DISCUSSION……59

CONCLUSION……60

APPENDIX……61

REFERENCES…….63

INTRODUCTION –

Milk, a highly nutritious and widely consumed dietary staple, plays an indispensable role in human nutrition due to its rich composition of proteins, fats, carbohydrates, vitamins, and minerals. However, the increasing demand for prolonged shelf-life and aesthetic appeal has led to the rampant use of artificial preservatives and adulterants in commercial milk production. Compounds such as potassium sorbate and natamycin are commonly employed for their antimicrobial properties. Yet, their unregulated or excessive usage poses serious public health concerns, especially in the context of antimicrobial resistance (AMR). Antimicrobial resistance has emerged as a global health crisis, undermining the effectiveness of standard treatments and facilitating the persistence and spread of resistant pathogens. Furthermore, ingesting adulterated milk compromises food safety, and routine consumption may have cumulative toxicological effects.

In India, the **Food Safety and Standards Authority of India (FSSAI)** serves as the apex body for ensuring the safety and hygiene of food products, including milk and dairy items. FSSAI establishes **Maximum Residue Limits (MRLs)** for preservatives in food, aiming to protect consumers from adverse effects and to prevent the misuse of chemical additives. Despite these regulations, several studies have indicated that preservative levels in some commercial milk samples may exceed the permissible limits, leading to potential health hazards. This is especially critical in unregulated or semi-regulated milk supply chains, including local dairies and street vendors.

Furthermore, under international food safety frameworks such as **Hazard Analysis and Critical Control Points (HACCP)**, identifying critical points in milk processing where contamination or preservative misuse may occur is essential. HACCP emphasizes preventive strategies and risk-based approaches to control biological, chemical, and physical hazards in food production systems. The integration of FSSAI standards with HACCP practices enhances the reliability of food safety systems and ensures compliance with both national and global benchmarks.

An emerging dimension of food safety is the impact of artificial preservatives on microbial ecosystems, particularly their role in the **emergence of antimicrobial resistance (AMR)**. Continuous exposure to sub-inhibitory levels of preservatives can induce adaptive responses in food-borne bacteria, including **Escherichia coli** and **Staphylococcus aureus**, leading to the development of cross-resistance to clinical antibiotics. This raises significant public health concerns, as AMR strains can enter the

human body through the consumption of contaminated milk, further complicating infection treatment.

This study aims to critically evaluate the **antimicrobial resistance patterns of bacterial isolates from milk samples**, with a focus on the **presence and concentration of artificial preservatives** such as potassium sorbate and natamycin. By correlating laboratory findings with **FSSAI permissible limits** and **HACCP-based risk assessments**, the project seeks to provide a comprehensive understanding of how preservative use may contribute to microbial resistance. The findings will not only contribute to scientific literature but also offer actionable insights for improving food quality assurance, public health policies, and consumer awareness in India and beyond.

This study aims to investigate the influence of selected artificial preservatives and adulterants on antimicrobial resistance patterns in bacterial isolates obtained from raw (shed) and commercially available milk. It also integrates both wet lab techniques, such as Minimum Inhibitory Concentration (MIC) testing and biochemical profiling, with computational approaches like molecular docking and predictive modeling. Through this multidisciplinary approach, the study seeks to generate robust data that can inform food safety regulations and raise awareness about the silent but critical threat posed by AMR arising from food sources.

LITERATURE REVIEW

Milk, a nutritionally rich fluid, is widely consumed across the globe. Its high nutrient content, while beneficial for human health, also makes it a suitable medium for microbial growth. Consequently, milk is often treated with artificial preservatives to ensure its shelf life and microbial safety. However, the long-term use and consumption of such preservatives raise concerns regarding antimicrobial resistance (AMR) and potential health hazards.

Several studies have documented the frequent use of **preservatives such as potassium sorbate and natamycin** in milk and dairy products to inhibit yeast, mold, and certain bacteria (Gautam et al., 2021). While regulatory agencies generally recognize these compounds as safe (GRAS) when used within permissible limits, emerging evidence suggests that prolonged exposure may induce bacterial adaptation and resistance (El-Sayed et al., 2020).

Antimicrobial resistance in foodborne bacteria is a global concern, with the World Health Organization recognizing AMR as a major threat to food safety. A study by Bhattacharjee et al. (2019) highlighted the resistance patterns of *E. coli* and *Staphylococcus aureus* isolated from milk samples, indicating that even non-therapeutic exposure to preservatives might select for resistant strains over time.

The adulteration of milk with chemical agents, either to mask spoilage or to alter composition, further complicates the problem. Substances like detergent, urea, and formalin are sometimes illegally added, and their residues can influence microbial behavior and resistance profiles (Kumar & Sharma, 2018). These additives not only pose toxicological risks but may also interfere with the antimicrobial susceptibility of bacteria by altering pH or damaging cellular structures.

Research using **Minimum Inhibitory Concentration (MIC) assays** and molecular docking studies has shown that preservatives such as potassium sorbate may interact with bacterial enzymes, contributing to adaptive resistance mechanisms (Singh et al., 2022). Computational tools also allow the prediction of resistance gene activation, offering insights into how sublethal preservative exposure may lead to AMR.

Emerging alternatives like **plant-based antimicrobials and probiotics** are being explored to mitigate the issue, but artificial preservatives remain widely used due to their cost-effectiveness and ease of application. Therefore, continuous surveillance and a deeper understanding of their role in AMR are crucial, especially in countries where milk preservation methods may lack stringent regulatory oversight.

Recent advances in **in vitro** and **in silico** methodologies have shed light on the mechanisms of preservative-induced resistance. Minimum Inhibitory Concentration (MIC) assays are widely used to quantify bacterial resistance levels, while molecular docking studies suggest that compounds like potassium sorbate may bind to key microbial enzymes, thereby disrupting metabolic pathways and prompting compensatory resistance responses (Singh et al., 2022). Computational tools such as predictive modeling and resistance gene mapping are now being utilized to identify how preservative exposure may trigger efflux pumps or gene expression related to AMR. Alternative strategies, such as plant-derived antimicrobial compounds, bacteriocins, and probiotics, are currently being researched as more sustainable approaches to milk preservation. Nonetheless, artificial preservatives continue to dominate due to their lower cost, extended shelf stability, and ease of industrial integration. As a result, the balance between food safety, microbial control, and public health risk remains precarious.

Given these complexities, there is a pressing need for continuous **surveillance of preservative usage** and deeper investigation into its microbial consequences. Countries like India, with rapidly expanding dairy industries, must especially uphold and enforce FSSAI guidelines alongside international frameworks such as Codex Alimentarius and HACCP (Hazard Analysis and Critical Control Points) to monitor preservative usage and assess its impact on bacterial ecology.This project seeks to bridge this knowledge gap by assessing AMR patterns in bacterial isolates from milk treated with common preservatives. By correlating preservative concentrations with bacterial resistance via laboratory and computational approaches, the study aims to comprehensively evaluate the microbiological and public health risks associated with these additives.

METHODS AND MATERIALS

1. COLLECTION OF SAMPLES AND ISOLATION OF MICROORGANISMS

- SAMPLE - At first, shed milk was collected from a nearby shed.

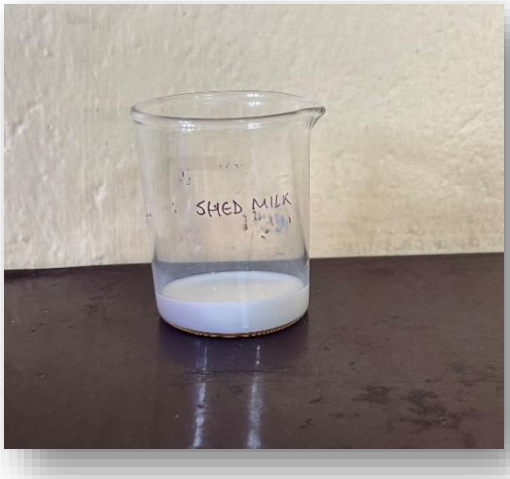


Fig.1: Sample of shed milk

- **Isolation of microorganisms-** After that, 20 ml of nutrient broth was prepared and. autoclaved the media at **121°C** and **15 psi** pressure for **15 minutes** to sterilize it completely.

INGREDIENTS	AMOUNT in gm
Peptone	0.2g
Meat extract	0.2g
Sodium chloride	0.1g
Agar	0.45g

Serial Dilution of Shed Milk Sample (Up to 10^3 Dilution):

- Label three sterile test tubes or microcentrifuge tubes as **10^1**, **10^2**, and **10^3**.
- Add **900 µL** of sterile **0.9% NaCl solution** (normal saline) into each tube.
- Using a sterile micropipette or pipette:
    - Add 100 µL of the shed milk sample to the **first tube (10^1)** containing 900 µL of NaCl.
    - Mix thoroughly by pipetting up and down or by vortexing gently.
- From the 10^{-1} dilution tube:
    - Take **100 µL** and add it into the second tube (10^2) containing 900 µL NaCl. Mix thoroughly again.
- From the 10^{-2} dilution tube:
    - Take **100 µL** and add it into the third tube (10^3) containing 900 µL NaCl. Mix thoroughly.
- Now, you have **10^{-1}**, **10^{-2}**, and **10^{-3}** serial dilutions ready for plating.

Plating the Diluted Samples:

- Using a sterile pipette tip, take **100 µL** from the **10^3 dilution tube**. Spread the 100 µL sample evenly on a solidified NA plate's surface using a sterile L-shaped or glass spreader.
- Allow the sample to absorb into the agar. Incubate the plates **inverted** at **37°C** for **24 hours** (or until visible colonies).

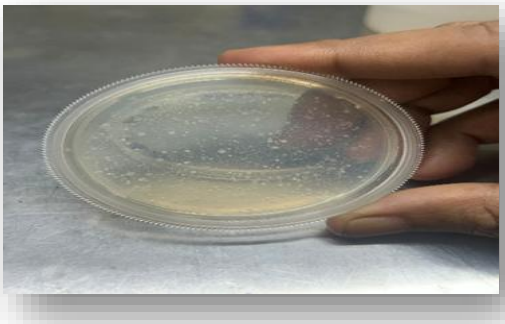


Fig.2: Growth of microorganism

Pure colony Isolation –

- As there was a presence of two types of colonies, one small and another big colony, a pure isolation procedure was performed.
- Using a sterile inoculating loop, pick individual colonies and streak them onto fresh nutrient agar plates to obtain pure cultures.
- Incubate the streaked plates at 37°C for another 24 hours.

RESULTS –

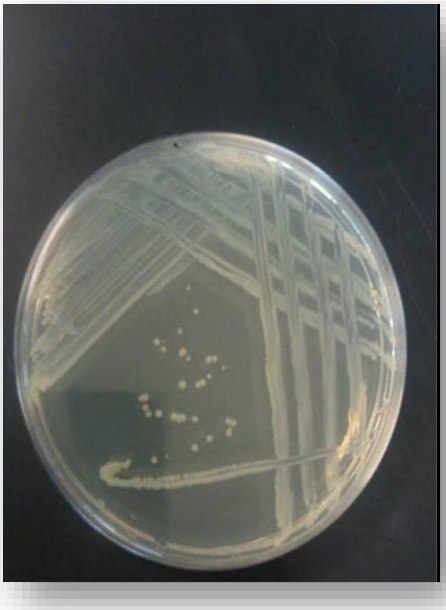


Fig.3: Pure culture 1

Fig.4: Pure culture 2

- After this, biochemical tests were performed on pure colonies to identify bacterial species.

BIOCHEMICAL TESTS-

Catalase Test–

- A loopful of bacterial colony was placed on a clean slide.
- A drop of 3% hydrogen peroxide was added.
- Immediate bubble formation indicated a positive catalase reaction.

RESULTS –

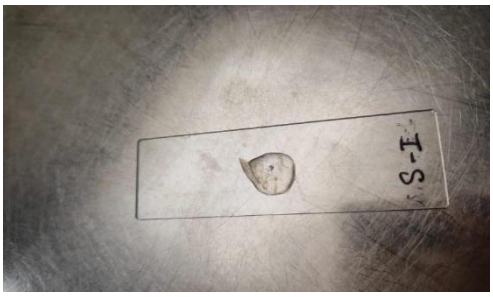


Fig.5(a): sample 1 catalase test

Fig.5(b): sample 2 catalase test

Indole Test-

- The bacteria were inoculated into tryptone broth and incubated at 37°C for 24-48 hours.
- After incubation, 5 drops of Kovac's reagent were added carefully along the sides. Pink colour rings form.

RESULTS – The first test tube showed a negative result, and the second test tube showed a positive result.

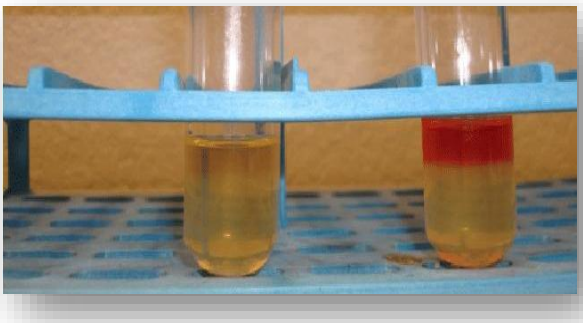


FIGURE 6: INDICATING THE TWO RESULTS

Simmon Citrate Test:

- The pure colony was streaked into an agar slant.
- After 24-48 hours of incubation, a colour change from green to blue indicated positive citrate utilization.

RESULTS – The first test tube showed a positive outcome, and the second test tube showed a negative result.

FIGURE 7 INDICATING THE RESULTS

Methyl Red Test (MR)

- The pure colony was incubated in MR-VP broth and incubated at **37°C for 48 hours**.
- Add a few drops of **Methyl Red indicator** after incubation. Observe the colour change to red.

RESULTS – Both the bacterial cultures showed positive results.

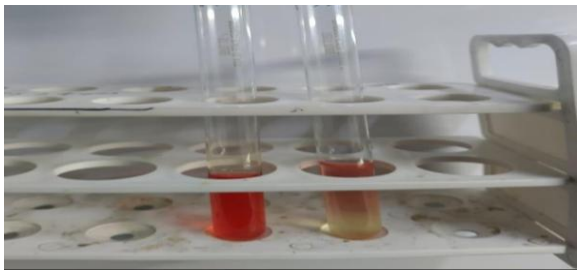


FIGURE 8: BOTH INDICATING POSITIVE RESULTS

Voges-Proskauer Test (VP)

- After incubation in MR-VP broth, add:
  - 0.6 mL of **alpha-naphthol**.
  - 0.2 mL of **KOH solution**.
- Mix gently and leave for 15–30 minutes.
- Observe colour change.

RESULTS –

The first test tube showed a positive result, and the second test tube showed a negative result.

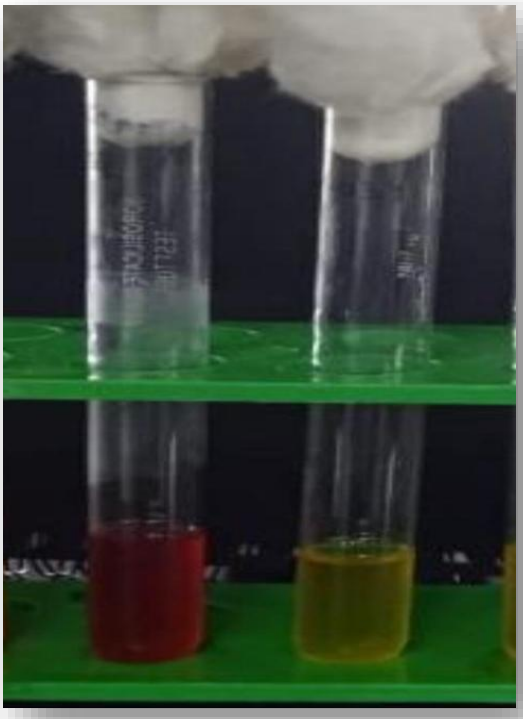


FIGURE 9: INDICATING TWO DIFFERENT RESULTS

Urease Test-

- The colony is inoculated onto the surface of **urea agar slant**.
- And then it was incubated at **37°C** for **24–48 hours**.
- Observe for colour change.

Gram staining-

1. A clean glass slide was taken, and a thin smear of bacterial culture from the milk sample was prepared using a sterile inoculating loop.
2. The smear was air-dried and then heat-fixed by gently passing it over a flame.
3. The slide was flooded with **crystal violet** stain for **one minute** and then gently rinsed with distilled water.
4. **Gram's iodine** solution was added and left for **one minute** to act as a mordant, followed by gentle rinsing.
5. The slide was then decolorized with **95% ethanol** for **15–20 seconds** and immediately rinsed with water to prevent over-decolorization.
6. **Safranin** counterstain was applied for **one minute**, and the slide was rinsed and air-dried.
7. The stained slide was observed under a **light microscope** using a **100x oil immersion objective**.

RESULTS –

Fig.10(a): Pink colour cells were visible

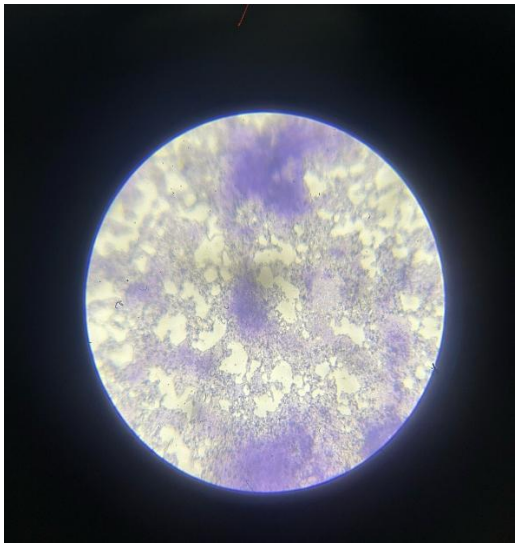


Fig.10(b): Purple colour cells were visible

Observation:

- **Purple-coloured cells** indicated **Gram-positive bacteria**.
- **Pink/red-coloured cells** indicated **Gram-negative bacteria**.

Based on Gram staining results:

- Isolates showing **purple colour** were identified as Gram-positive bacteria (e.g., ***Staphylococcus aureus***).
- Isolates showing **pink colour** were identified as Gram-negative bacteria (e.g., ***Escherichia coli***).

RESULTS –

COLONY	***Circular, Smooth, Mucoid***	***Large, circular, smooth***
CELL MORPHOLOGY	***Rod***	***Cocci***
GRAM STAINING	***-ve***	***+ve***
CATALASE	***-ve***	***+ve***
INDOLE TEST	***+ve***	***-ve***
UREASE TEST	***-ve***	***+ve***
SIMMON CITRATE TEST	***-ve***	***+ve***
MR TEST	***+ve***	***+ve***
VP TEST	***-ve***	***+ve***
DEXTROSE TEST	***+ve***	***+ve***

Hence, the pure cultures collected were indicated as ***Escherichia coli*** and ***Staphylococcus aureus.***

Biochemical Estimation of Nutrients in Milk Samples

Carbohydrate Test –

Materials Required:

- Glucose (Standard)
- 2.5 N Hydrochloric acid (HCl)
- Sodium carbonate (solid)
- Phenol (5%)
- Concentrated sulfuric acid (H_2SO_4)
- Water bath
- Spectrophotometer (490 nm)

Procedure:

1. Preparation of Standard Curve:

- 0.1 mL, 0.2 mL, 0.3 mL, 0.4 mL, and 0.5 mL of the standard glucose solution were transferred into individual test tubes. And distilled water to make 1 mL.

2. Sample Handling:

- Take 1 mL of each milk sample (shed milk and commercial milk) into separate labelled test tubes.

3. Reagent Addition for Colour Reaction:

- 0.5 mL of a 5% phenol solution was added to each tube, in standards and samples.
- 2.5 mL of concentrated sulfuric acid was added to each test tube and mixed thoroughly.

4. Incubation for Colour Development:

- Before the water bath, it was allowed to rest for 10 minutes.
- Gently shake the tubes again and place them in a water bath maintained at 25–30°C for 30 minutes to facilitate colour formation.

5. Absorbance Measurement:

- Absorbance at 490 nm using a spectrophotometer was used here.
- Use a reagent blank (containing all reagents except glucose) as the reference.

STANDARD	SAMPLES
Fig.11(a): Standard samples	Fig.11(b): Unknown samples

RESULT- (TABLE 2)

SL.NO.	SAMPLE	DISTILLED WATER	PHENOL	SULPHURIC ACID	ABSORBANCE
Blank	-	1 mL	500 µL	2.5 mL	-
0.1	100 mL	900 mL	500 µL	2.5 mL	0.205
0.2	200 mL	800 mL	500 µL	2.5 mL	0.215
0.3	300 mL	700 mL	500 µL	2.5 mL	0.239
0.4	400 mL	600 mL	500 µL	2.5 mL	0.263
0.5	500 mL	500 mL	500 µL	2.5 mL	0.266
Shed milk	500 mL	500 mL	500 µL	2.5 mL	1.416
Commercial milk	500 mL	500 mL	500 µL	2.5 mL	1.443

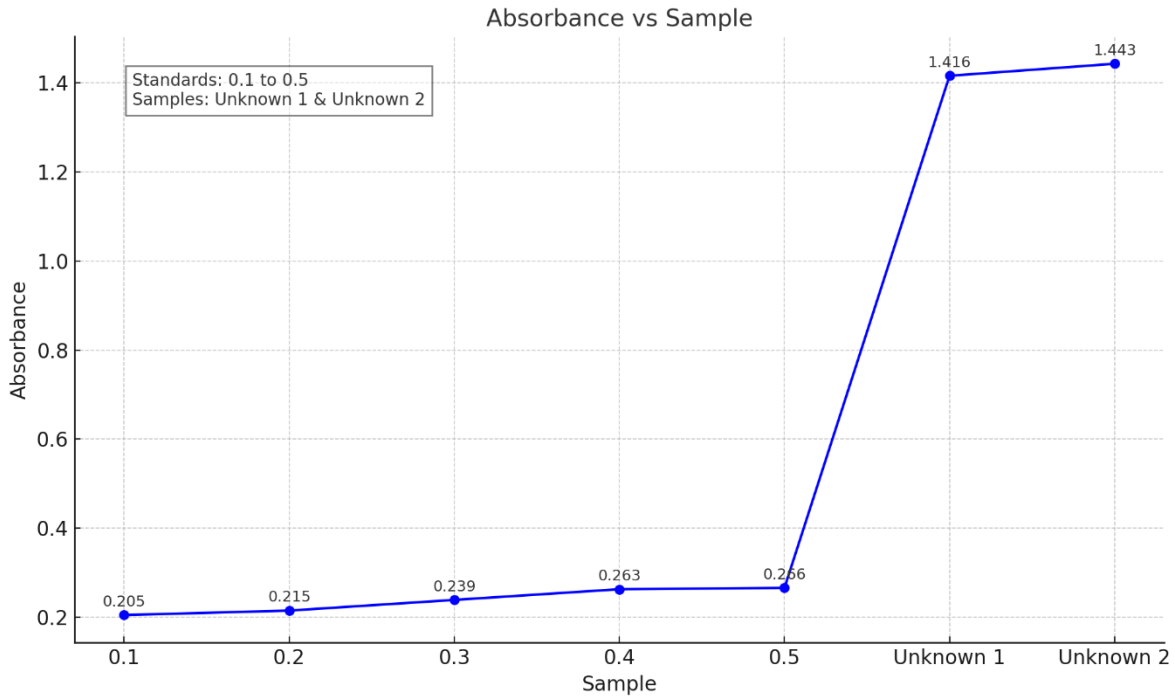


Fig.12: Absorbance of Carbohydrate

The **absorbance values of the milk samples** (1.416 and 1.443) are significantly higher than the standards, indicating **high carbohydrate content**, which is expected in milk.

Calculation of % Carbohydrate

Percentage of total carbohydrate present = **(x/0.1) ×100/ Sample volume (mL)**

where:

- x = equivalent glucose concentration for your sample (from standard curve).
- **0.1** = known standard volume (mL).
- **100** = conversion factor to percentage.
-

From the **standard data**:

By using a linear equation (y = mx + c).

We can approximate the slope (m): **m≈ (0.266 – 0.205) / (0.5 – 0.1) = 0.061/0.1 = 0.1525**

The y-intercept (ccc) can be estimated by:

C = 0.255 – (0.1525 ×0.1) = 0.205−0.01525 = 0.18975

Use the calibration equation:

Absorbance = **0.1525 × Glucose (mL) + 0.18975**

Solve for glucose:

Glucose (mL) = **(Absorbance − 0.18975) / 0.1525**

- **SHED MILK** –

  Glucose (mL) = **1.416 – 0.18975 / 0.1525 = 1.22625 / 0.1525 ≈ 8.04 mL**

- **COMMERCIAL MILK –**

  Glucose (mL) = **1.443 - 0.18975 / 0.1525 = 1.25325 / 0.1525 ≈8.21Ml**

Percentage of Total Carbohydrate

Since 100 mL of sample solution was used:

- **Shed Milk % Carbohydrate**:

  % Carbohydrate = (8.04/0.1) ×100 / 100 = 804 / 100 = 8.04%

- **Commercial Milk % Carbohydrate**

  % Carbohydrate = (8.21/0.1) ×100 / 100 = 821 / 100 = 8.21%

OVERALL –

- ✓ **Shed milk:** 8.04% total carbohydrates
- ✓ **Commercial milk:** 8.21% total carbohydrates

Sugar Test –

Three Eppendorf tubes were taken:

- One tube was used as a **standard control** (glucose solution).
- Two tubes were used for the **unknown samples** (shed milk and commercial milk). Into the **standard control tube**, 500 µL of glucose solution was added. To the standard, **0.1 mL of Fehling's A** and **0.1 mL of Fehling's B** solutions were added slowly and mixed. Similarly, **500 µL of shed milk** and **500 µL of commercial milk** were taken into the other two tubes, respectively, and the amount was slowly increased.

i. Before adding anything, the empty weight of each Eppendorf tube was measured and recorded.
ii. To each sample, **0.1 mL of Fehling's A** and **0.1 mL of Fehling's B** solutions were added slowly.

iii. **Methylene blue** was added **dropwise** to each Eppendorf tube containing the samples until a visible **colour change** started, from blue to the beginning of **red precipitate formation**.

iv. The tubes were allowed to **rest undisturbed for 10–20 minutes** to ensure complete precipitation of the **brick-red cuprous oxide**.
v. After completion, the amount of sugar present was calculated based on the precipitate formation and compared with the standard control.

CALCULATION -

Sugar concentration for commercial milk –

The volume of standard glucose solution used for titrating Fehling's solution = **V1**

The volume of the unknown glucose solution used for titrating Fehling's solution = **V2**

Amount of glucose in the unknown glucose solution = **M×V1/V2**

The strength of the unknown glucose solution = **4× m × V1/V2**

- Weight of sample + Eppendorf = 2.127
- Weight of empty Eppendorf = 1.208
- Final weight = 0.919
- V1(standard) = 1.5 mL
- V2 (sample) = 2 mL

➢ M = Final weight of the Eppendorf × V2 = 0.919 × 2 = 1.838

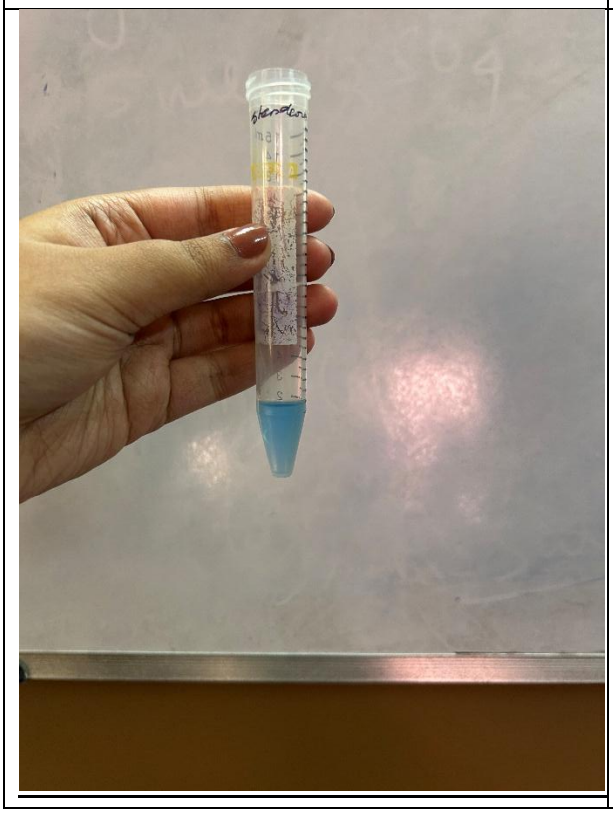
STANDARD	SAMPLE
	

Fig.13(a): Sugar results (commercial milk)

RESULTS-

➢ Amount of unknown glucose = M × V1/V2

= 1.838 × 1.5/2

= 1.37

➢ Strength of unknown glucose sample = 4× m × V1/V2

= 4×1.838×1.5/2 = **5.514**

Sugar concentration for shed milk –

- Weight of sample + Eppendorf = 2.283
- Weight of empty Eppendorf = 1.256
- Final weight = 1.027
- V1(standard) = 1.45 mL
- V2 (sample) = 2.5 mL

➢ M = Final weight of the Eppendorf × V2 = 1.027 × 2.5 = 2.567

➢ Amount of unknown glucose = M × V1/V2
= 2.567 × 1.45/2.5
= 1.49

➢ Strength of unknown glucose sample = 4× m × V1/V2
= 4×2.567×1.45/2.5 = **5.955**

RESULTS-

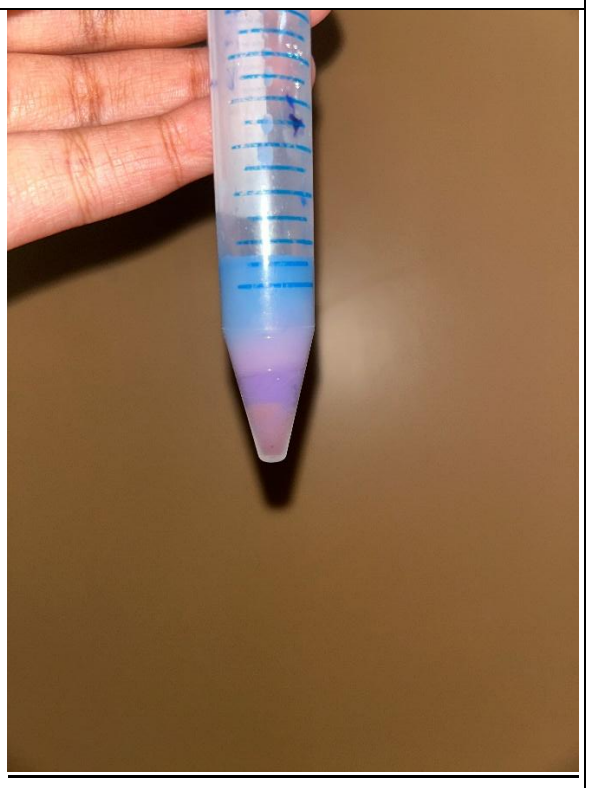
STANDARD	SAMPLE
	

Fig. 13 (b): Sugar results (shed milk)

Protein Estimation (Lowry's Method)

Materials Required

- **Milk samples** (shed and commercial)
- **Bovine Serum Albumin (BSA)** – as a protein standard
- **Lowry's Reagent A** (Alkaline Copper Sulfate)
- **Folin–Ciocalteu reagent** (1N, diluted)
- **Distilled water**
- **Volumetric flasks, test tubes**
- **Spectrophotometer (set at 660 nm)**
- **Pipettes and micropipettes**
- **Water bath or incubator**

Reagent Preparation

1. **Standard Protein Solution**:
    - Dissolve 100 mg of BSA in 100 mL of distilled water to make a 1 mg/mL stock.
    - Prepare working standards of 20, 40, 60, 80, and 100 µg/mL by dilution.
2. **Lowry's Reagent (Reagent A)**:
    - Mix: 2% Na_2CO_3 in 0.1N NaOH + 0.5% $CuSO_4$ + 1% potassium sodium tartrate.
    - Combine just before use.
3. **Diluted Folin-Ciocalteu Reagent**:
    - Dilute commercial reagent with distilled water in a 1:1 ratio.

Procedure -

1. Standard and Sample Preparation

- Take **0.2 to 1 mL** of each BSA standard in separate test tubes.
- Add **0.2 mL** of shed milk and commercial milk samples into two additional tubes.
- Make the volume in each tube up to **1 mL with distilled water**.

2. Addition of Reagents

- **5 mL of Lowry's reagent (Reagent A)** was added to all tubes (standards and samples.
- After that, there were 10 minutes of incubation.
- **0.5 mL of diluted Folin–Ciocalteu reagent** was given to each test tube and **mixed immediately**.

3. Incubation and Colour Development

- Incubate all tubes in a **dark place or water bath** at **room temperature (25–30°C) for 30 minutes** to allow blue colour development.

4. Measurement

- Measure the **absorbance at 660 nm** in a spectrophotometer.
- Use a blank (with all reagents but no protein) to calibrate the instrument.

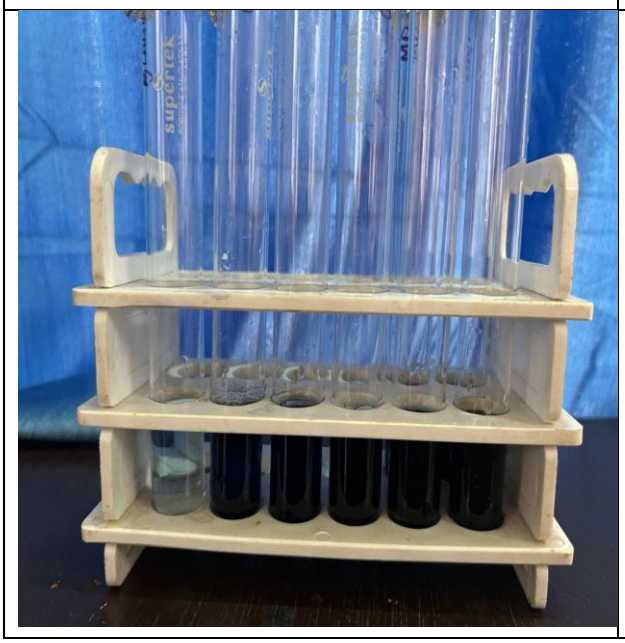
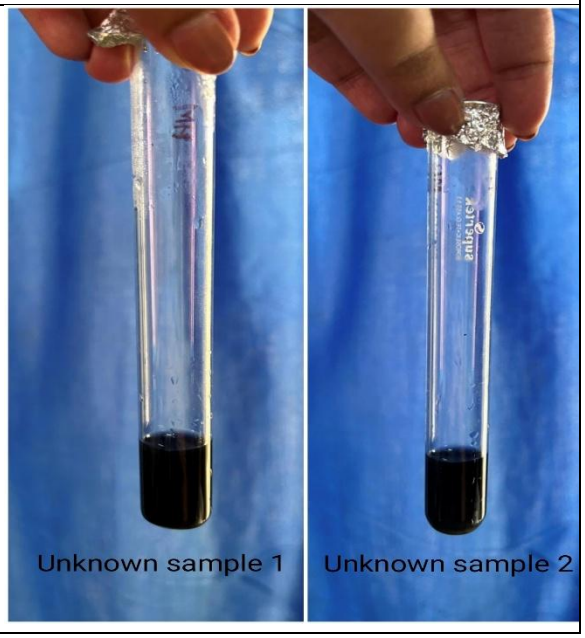
STANDARD	SAMPLES
	

Fig.14(a): Standard Protein samples

Fig.14(b): Unknown samples

Standard Curve –(TABLE 3)

Concentration (µg/mL)	Absorbance
0 (Blank)	0.000
20	0.130
40	0.260
60	0.395
80	0.530
100	0.665
Sample 1	0.480
Sample 2	0.600

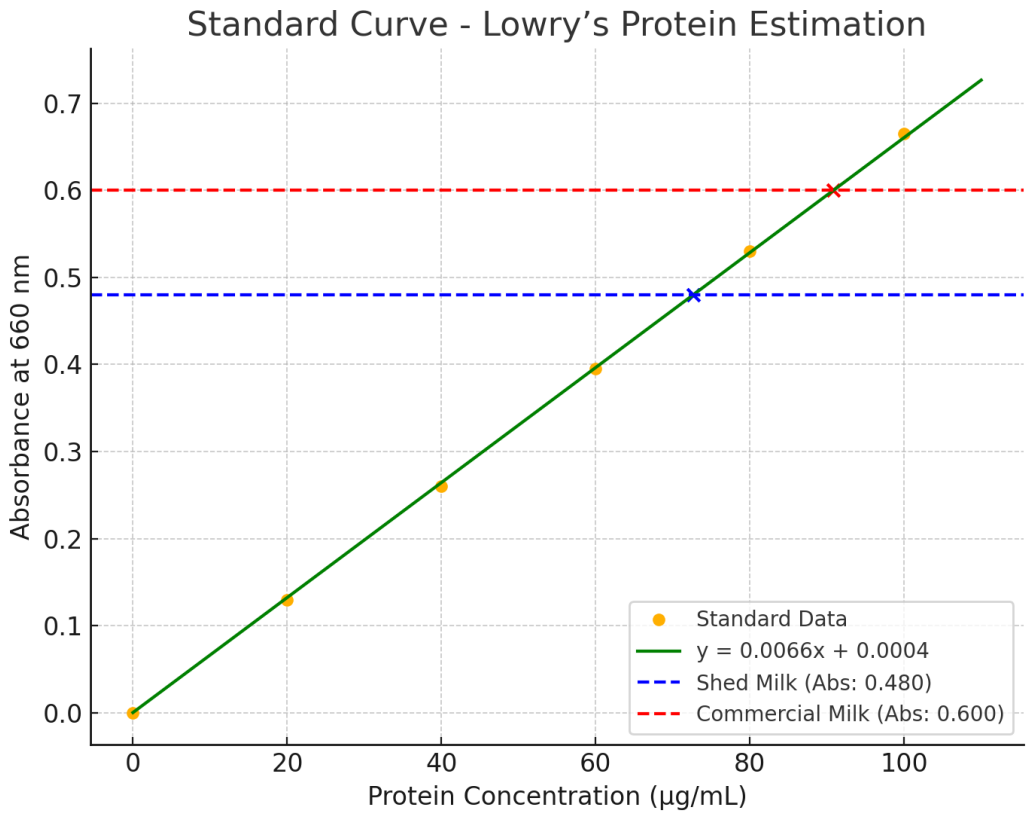


Fig.15: Standard graph

Standard Curve Equation:

- Taking two points: (20,0.130 and 100,0.665)

Step 1: Finding the slope

m = (0.665– 0.130/ 100 – 20) = 0.535/80 = 0.0066

Step 2: Using one point to solve for b (for this, one point is used)

One point (20,0.130)

$0.130 = 0.0066 \times 20 + b \Longrightarrow b = 0.134 - 0.130 = 0.004$

Final equation = y = 0.0066x + 0.0004

Here:

- y is the **absorbance**
- x is the **protein concentration in µg/mL**
- 0.0066 is the **slope (m)** of the line
- 0.0004 is the **y-intercept (b)** — the absorbance value when there is no protein

Sample Calculations

Shed Milk Sample:
Absorbance = 0.480
Using the equation:
0.480 = 0.0066x + 0.0004
x = (0.480 - 0.0004) / 0.0066 ≈ 72.67 µg/mL = 0.0727 mg/mL

Commercial Milk Sample:
Absorbance = 0.600
x = (0.600 - 0.0004) / 0.0066 ≈ 90.82 µg/mL = 0.0908 mg/mL

Conclusion

- **Commercial milk** showed a **higher protein content (0.0908 mg/mL)** than **shed milk (0.0727 mg/mL)**.
- This may be due to standardization or adding milk solids during commercial processing.

FAT ESTIMATION –

i. 20 mL each of shed milk and commercial milk were taken separately in two beakers. (shed milk was 7.1 and commercial milk was 6.51)

ii. A **50:50 dilution** of distilled water and 1% HCl solution was prepared. The diluted acid was slowly added to the milk samples while pH monitoring.

iii. Acid was added until the pH dropped to around **4** or **lower**, indicating the beginning of milk coagulation.

iv. After approximately **five minutes**, coagulation started visibly. The milk samples were then heated to **50–60°C** to enhance coagulation.

Fig.16(a): Milk coagulation

Fig.16(b): Coagulated milk

v. After sufficient coagulation, the milk samples were filtered to separate the solid curd from the filtrate. The filtrate was collected carefully for fat estimation.

Fig.17(a): Separation of coagulated milk Fig.17(b): Extracted residue stored for fat test

vi. Empty Petri plates were weighed before use.

- Shed milk Petri plate weight before = **47.56 g**
- Commercial milk Petri plate weight before = **43.74 g**

Fat Extraction using Petroleum Ether:

Fig.18(a) and 18(b): Fat Separation of commercial and shed milk

<u>For shed milk:</u>

15 mL of petroleum ether was added to the filtrate.

<u>For commercial milk:</u>

10 mL of petroleum ether was added.

AFTER EXTRACTION OF FAT –

- The fat was separated into a beaker, and the residue was discarded.
- After that, the plates were kept in a hot air oven so that they would get dry, and we could measure the amount of fat by subtracting the actual weight of the petri plate.

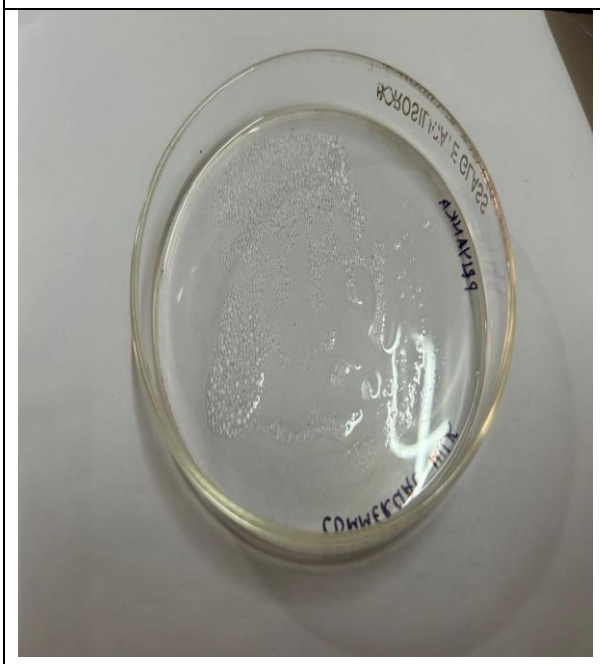
COMMERCIAL MILK	DISCARDED RESIDUE
	

Fig.19(a): Fat Extraction (Commercial milk)

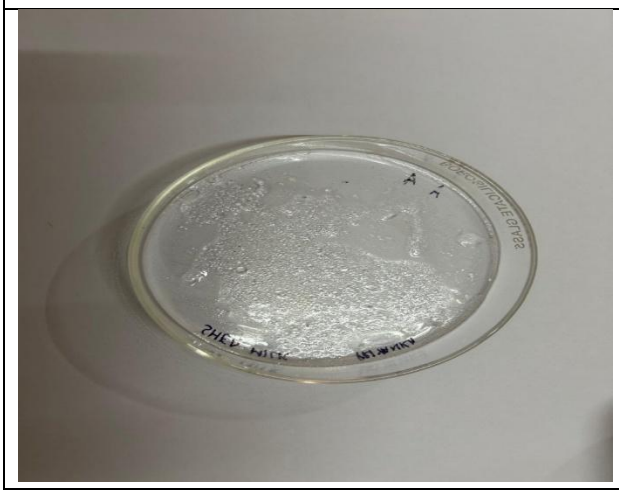
SHED MILK	**DISCARDED RESIDUE**
	

Fig.19(b): Fat Extraction (Shed milk)

Final Weighing:

- After the solvent was evaporated, the Petri plates were weighed again.
  - Shed milk Petri plate weight after = **47.68 g**
  - Commercial milk Petri plate weight after = **43.79 g**

Fat Content Calculation:

- Fat content was calculated by subtracting the initial weight of the Petri plate from the final weight after extraction:

For Shed Milk: 47.68 – 47.56 = 0.12g

For Commercial Milk: 43.79 – 43.74 = 0.05

RESULTS-

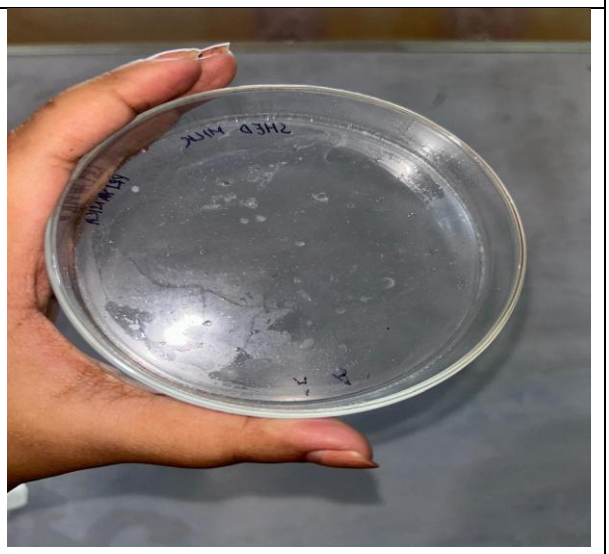
COMMERCIAL MILK	SHED MILK
	

Fig.20: Fat results

- **Fat content of Shed Milk** = **0.12 g**
- **Fat content of Commercial Milk** = **0.05 g**

PRESERVATIVE ESTIMATION IN COMMERCIAL MILK

i. A certain amount of milk was taken and heated to 60 degrees for 15 minutes.

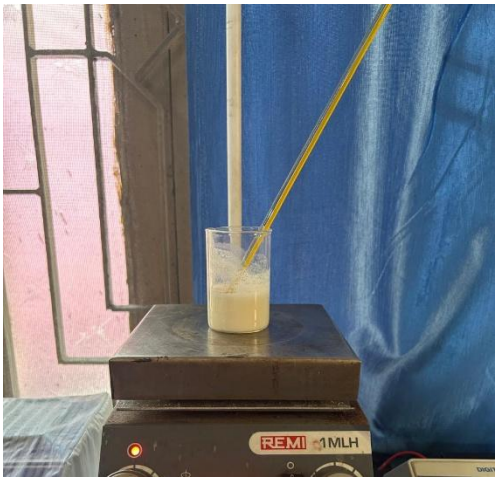


Fig.20(a): Boiling to coagulate

ii. HCL was added gradually so that the whole lump of casein was formed.

Fig.20(b): Coagulated milk

iii. After that, the whole milk was filtered off.

Fig.20(c) The lumps separation

iv. To the filtrate, sodium bicarbonate was added to neutralize the acetic acid by checking the pH up to 7, and then the whole solution was boiled for at least 15 minutes.

v. A whole bunch of precipitate was formed at the bottom and filtered off.

Fig.20(d): sodium bicarbonate precipitate

vi. The filtrate was taken, then subsequently assayed for potassium sorbate and natamycin.

Fig.20(e): Residue separation

Fig.20(f): Clear solution of milk

vii. Potassium sorbate and natamycin peaks were taken in the blank as distilled water.

viii. Potassium sorbate absorbance wavelength was 254nm, and natamycin was at – 318.2,304 and 296nm.

RESULTS –(TABLE 4)

Natamycin	**At 318.2nm**	**0.593**
Natamycin	**At 304nm**	**1.763**
Natamycin	**At 296nm**	**1.828**
Potassium sorbate	**At 254nm**	**1.955**

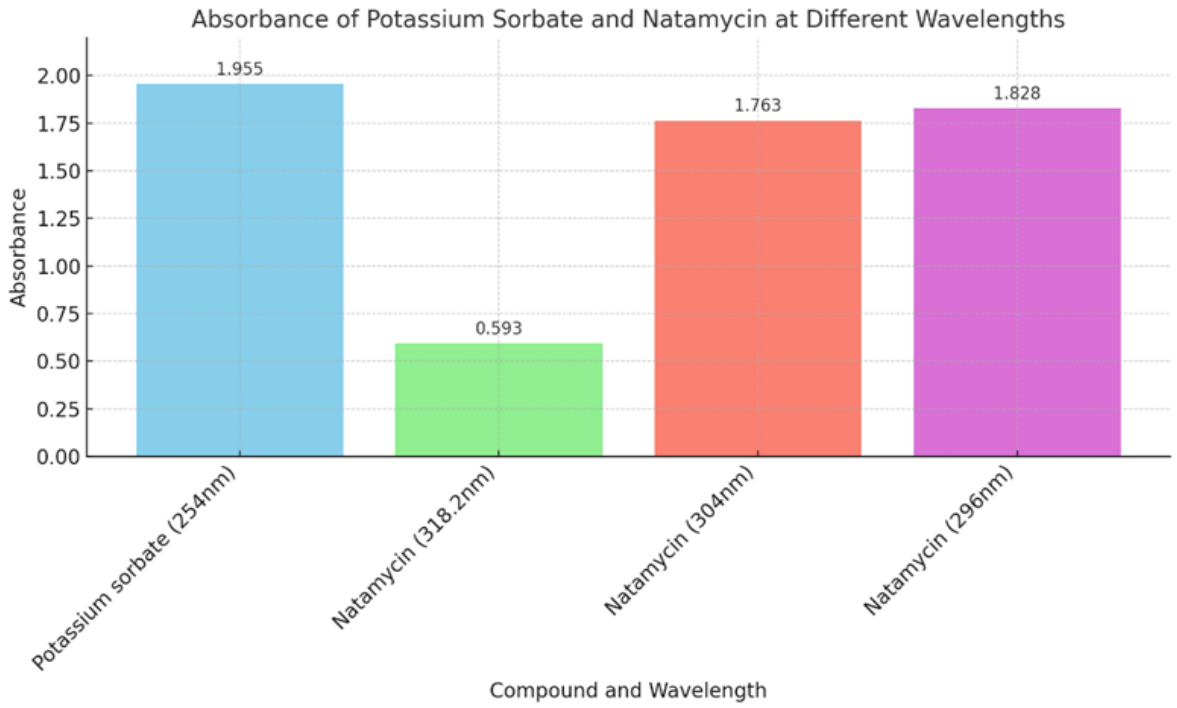


Fig.21: Absorbance in coloured bars

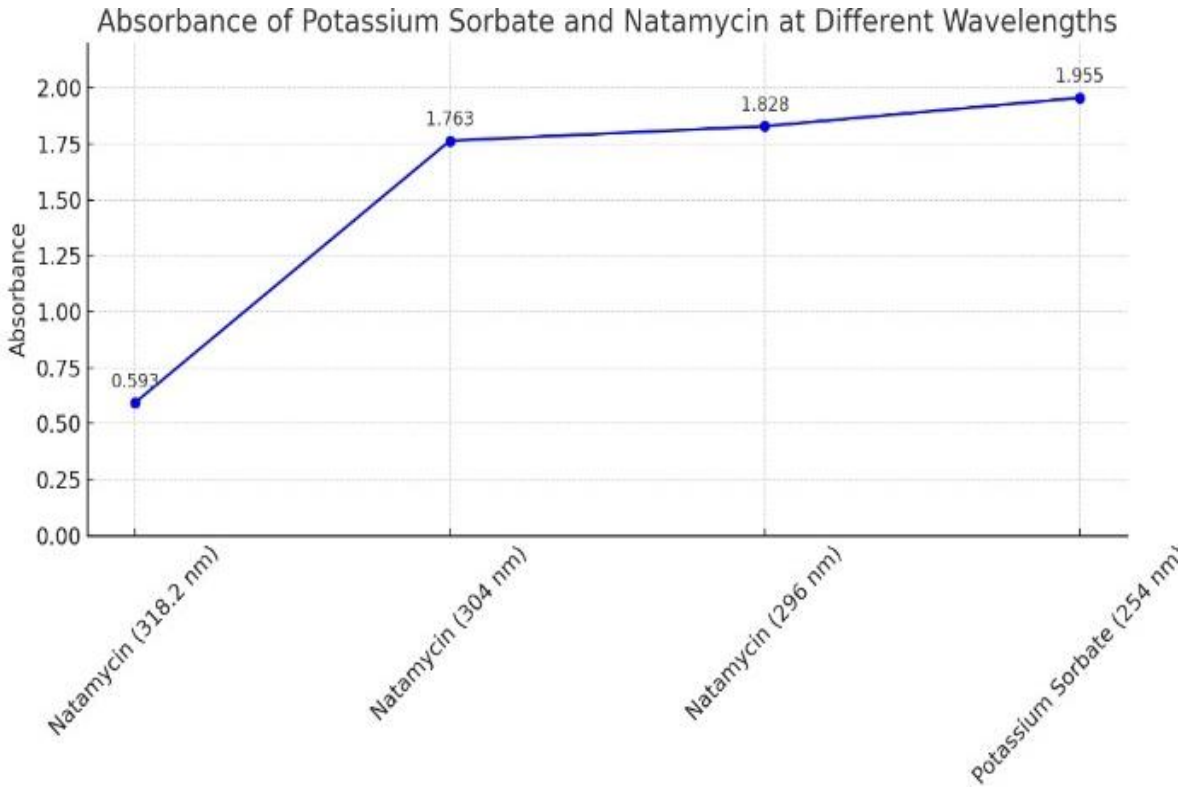


Fig.21(a): Absorbance graph

Standard Calibration Curve Preparation and Concentration Determination -

Potassium sorbate -

- **10, 20, 30, and 40 mg/L** of standard solutions were prepared for potassium sorbate.
- **A 254 nm** UV-Vis spectrophotometer was used, and the absorbance values were recorded. A calibration graph was plotted with **concentration (mg/L) on the X-axis** and **absorbance on the Y-axis**

A linear trendline was drawn, and the line equation was determined by linear regression. The resulting equation was:

TABLE 5 -

Concentration(mg/L)	Absorbance(254nm)
10	0.5
20	0.9
30	1.3
40	1.7

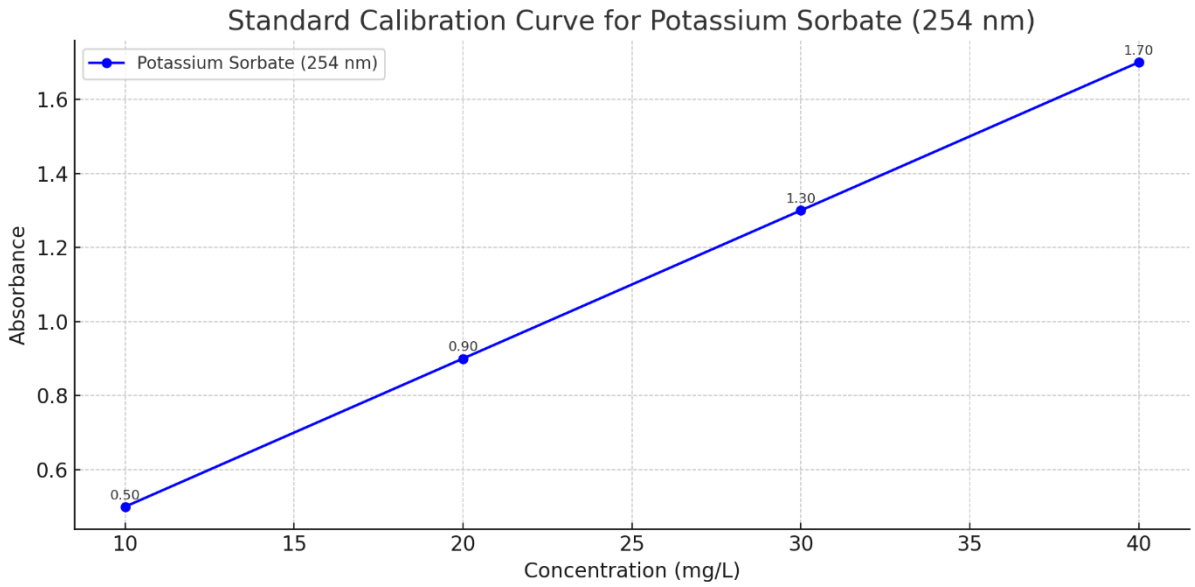


Fig.21(b): Graphical representation of Potassium sorbate

Using, Linear equation = y = mx + b

To find **x (concentration)**:
x = (y-b) / m

Where:

- *y* = absorbance
- *x* = concentration in mg/L
- *m = slope*
- *b = y-intercept*

For 254 nm

Step 1: Finding the slope

- Taking two points: (10,0.5 and 40,1.7)

m = (1.7 – 0.5/ 40 – 10) = 1.2/30 = 0.04

Step 2: Using one point to solve for b (for this, one point is used)

- One point (10,0.5) = 0.5 = 0.04(10) + b = 0.5 = 0.4+b

b = 0.5 – 0.4 = 0.1 = **Final equation = y = 0.04x + 0.1**

Natamycin –

- Standard solutions of natamycin were prepared in distilled water at known concentrations, like the potassium sorbate standards.
- Absorbance readings were recorded at three characteristic wavelengths of natamycin: **318.2 nm, 304 nm, and 296 nm**.
- Separate calibration curves were plotted for each wavelength using their corresponding absorbance data.

The following linear equations were obtained through regression analysis (TABLE 6):

Concentration(mg/L)	Absorbance(318.2nm)	Absorbance(304nm)	Absorbance(296nm)
10	0.37	0.536	0.529
20	0.54	0.872	0.858
30	0.71	1.208	1.187
40	0.88	1.544	1.516

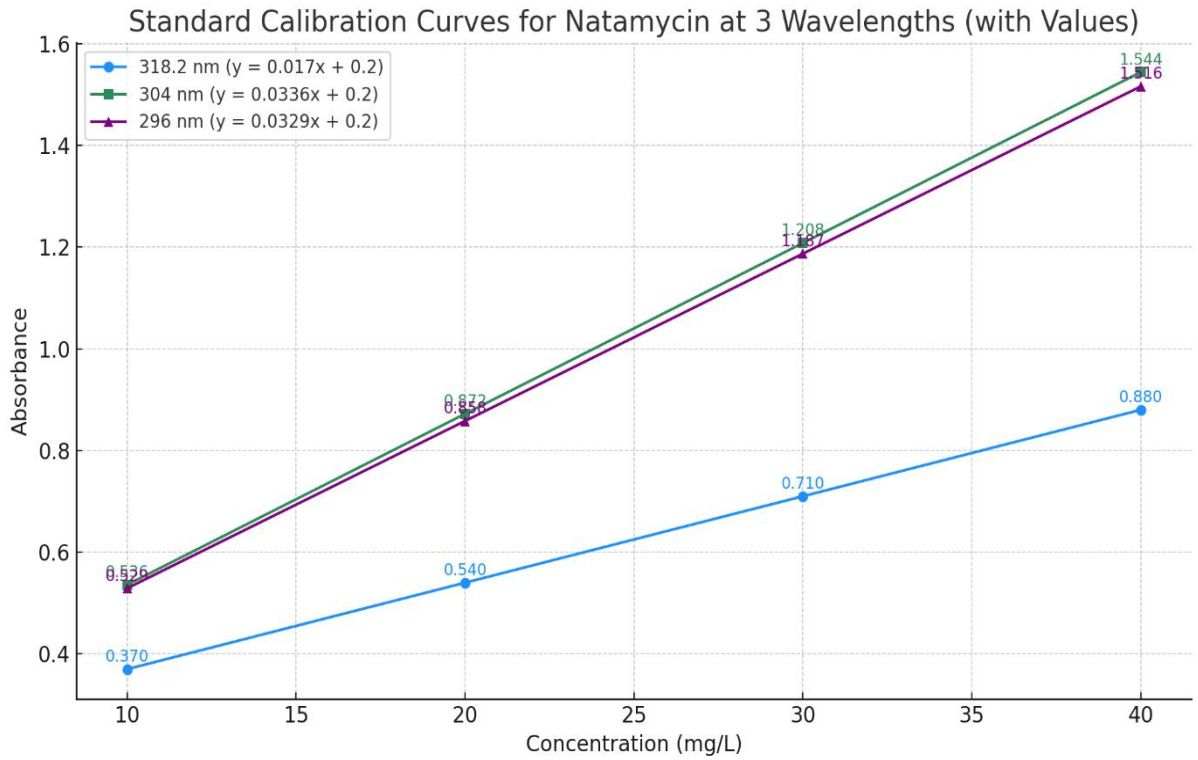


Fig.21(c): Graphical representation of Natamycin

In the same way, natamycin was calculated –

For 318.2 nm

Step 1: Finding the slope

- Taking two points (10,0.37 and 40,0.88)

  m = (0.88 – 0.37)/ (40 -10) = 0.51/30 = 0.017

Step 2: To find b

- Use one point to solve b (10,0.37)

  0.37 = 0.017(10) +b = 0.37 = 0.17 +b

  b = 0.37 – 0.17 = 0.2

Final equation = y = 0.017x + 0.2

For 304 nm

Step 1: Find the slope (m)

m = (1.544 -0.536/ 40-10) = 1.008/30 = 0.0336

Step 2: Find the intercept (b)

Using point (10, 0.536)

0.536 = 0.0336(10) +b = 0.536 = 0.336 +b

b = 0.536 – 0.336 = 0.2

Final equation = y = 0.0336x + 0.2

For 296 nm

Step 1: Find the slope (m)

m = (1.516 – 0.529 /40-10) = 0.987/30 = 0.0329

Step 2: Find the intercept (b)

0.529 = 0.0329(10) +b = 0.529 = 0.329 +b

b = 0.529 – 0.329 = 0.2

Final equation = y = 0.0329x + 0.2

So,

- **For Potassium Sorbate, At 254 nm: y = 0.04x + 0.1**
- **For Natamycin,**

  At 318.2 nm: **y = 0.017x + 0.2**

  At 304 nm: **y = 0.0336x + 0.2**

  At 296 nm: **y = 0.0329x + 0.2**

Using linear regression, the equations obtained were:

Using the formula -y = mx + b

1. Potassium Sorbate (254 nm)

Calibration Equation: y = 0.04x + 0.1

Sample Absorbance (y): 1.955

Using the formula x = (y-b) / m:

x = (1.955 - 0.1) / 0.04

x = 1.855 / 0.04 = 46.38 mg/L

2. Natamycin (318.2 nm)

Calibration Equation: y = 0.017x + 0.2

Sample Absorbance (y): 0.593

x = (0.593 - 0.2) / 0.017

x = 0.393 / 0.017 = 23.12 mg/L

3. Natamycin (304 nm)

Calibration Equation: y = 0.0336x + 0.2

Sample Absorbance (y): 1.763

x = (1.763 - 0.2) / 0.0336

x = 1.563 / 0.0336 = 46.51 mg/L

4. Natamycin (296 nm)

Calibration Equation: y = 0.0329x + 0.2

Sample Absorbance (y): 1.828

x = (1.828 - 0.2) / 0.0329

x = 1.628 / 0.0329 = 49.49 mg/L

5. Average Natamycin Concentration

Average = (23.12 + 46.51 + 49.49) / 3 = 119.12 / 3 = 39.71 mg/L

→ Average Natamycin Concentration: 39.71 mg/L

ANTIMICROBIAL RESISTANCE TEST –

Materials Required:

- Nutrient agar powder
- Distilled water
- Petri plates
- Inoculating loop
- Micropipette and sterile tips
- Sterile cork borer
- Bacterial cultures: *E. coli* and *S. aureus*
- Potassium sorbate
- Natamycin
- Sterile conical flasks
- Incubator
- Measuring cylinder
- Marker
- Scale/ruler for measuring zone diameter

Preparation of Solutions:

1. Preparation of Nutrient Agar:

- Dissolve the recommended amount of nutrient agar powder in distilled water (as per manufacturer's instructions).
- Boil until fully dissolved.
- Autoclaving at 121°C for 15 minutes for sterilization.
- Pour into sterile petri plates and allow to solidify at room temperature.

2. Preparation of Potassium Sorbate and Natamycin Stock Solutions:

- Weigh **1 g** of **potassium sorbate** and **1 g** of **natamycin** separately.
- Dissolve each in **10 mL** of distilled water to make **100 mg/mL** stock solutions.
- Prepare fresh and use immediately for best results.

3. Control Solution:

- Sterile distilled water (50 µL) as the **negative control**.

Procedure:

1. Inoculation of Bacteria:

- Prepare fresh bacterial cultures (*E. coli* and *S. aureus*).
- Spread **100 µL** of bacterial suspension evenly on nutrient agar plates using a sterile spreader.
- Let the plates dry for **5 minutes** at room temperature.

2. Well Diffusion Method:

- A **sterile cork borer was used** to punch **three wells** into the agar in each plate.
- After that, the agar plugs were placed without damaging the base.

3. Application of Test Solutions:

- To each well, **50 µL** of each preservative was added:
  - **Well 1:** Potassium Sorbate
  - **Well 2:** Natamycin
  - **Well 3:** Sterile distilled water (Control)

Fig.22: Potassium sorbate and Natamycin dissolved in distilled water

4. Incubation:

- Incubated at **37°C for 24 hours**.

5. Observation:

- After incubation, the **zones of inhibition were observed**.
- Measure the **diameter of inhibition zones** in millimetres (mm) using a scale.

RESULTS –

- ***E. coli*** **plate -**

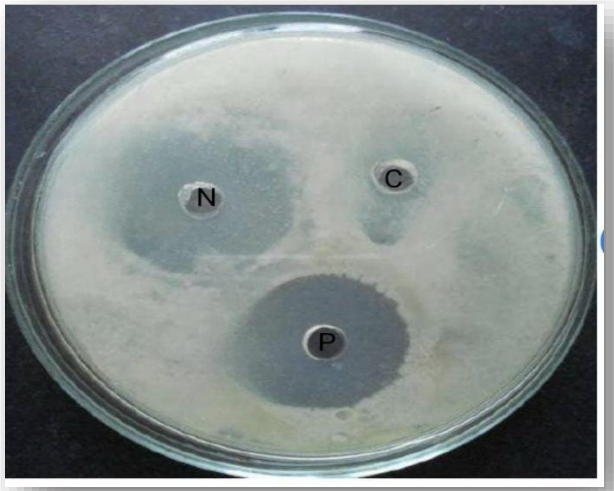


Fig.23(a): Zone of inhibition

- ***S. aureus*** **plate –**

Fig.23(b): Zone of Inhibition

Bacteria	Preservatives	Zone Of Inhibition
S. aureus	Potassium sorbate	18mm
S. aureus	Natamycin	20mm
S. aureus	Control (Sterile water)	0mm
E. coli	Potassium sorbate	14mm
E. coli	Natamycin	12mm
E. coli	Control (Sterile water)	0mm

Observations and Interpretations from Antimicrobial Resistance Test

1. ***Staphylococcus aureus***

- Potassium Sorbate (14 mm): Sensitive (S)
- Natamycin (20 mm): Sensitive (S)
- Control – Sterile Water (0 mm): No inhibition – Resistant (R)

Both potassium sorbate and natamycin are effective against S. aureus at the tested concentrations.

2. ***Escherichia coli***

- Potassium Sorbate (14 mm): Sensitive (S)
- Natamycin (12 mm): Sensitive (S)
- Control – Sterile Water (0 mm): No inhibition – Resistant (R)

Both potassium sorbate and natamycin effectively inhibit ***E. coli*** growth.

Potassium sorbate shows slightly higher inhibition compared to natamycin against ***E. coli.***

Natamycin exhibits a stronger inhibitory effect compared to potassium sorbate, indicating higher efficacy.

Both potassium sorbate and natamycin demonstrate antimicrobial activity against ***E. coli*** and ***S. aureus*** at the tested concentrations.

Natamycin appears to be more effective against ***S. aureus***, while potassium sorbate shows comparable or slightly better inhibition of ***E. coli.***

Assessment of Minimum Inhibitory Concentration for Potassium Sorbate and Natamycin

Preparation of Inoculum-15 mL of sterile Luria Bertani (LB) broth was prepared in a clean, sterile conical tube.

Composition	Amount in gm
Tryptone	0.15g
Yeast	0.075g
NaCl	0.15g

i. Using a sterile inoculation loop, **bacterial colonies** (previously isolated on nutrient agar plates) were picked and transferred into the LB broth.
ii. The inoculated broth was placed in a **shaker and incubator** at 37 °C and agitated at 150 rpm.
iii. The incubation continued until the bacterial culture's optical density (OD_{600}) reached **0.6**, indicating the log phase of bacterial growth. This culture was then used as the **standardized inoculum** for testing.

Preparation of Preservative Solutions

- **Potassium Sorbate** and **Natamycin** were selected as the preservatives to be tested for their antimicrobial activity.
-
- Based on their solubility:
    - **Potassium Sorbate**: 100 mg/mL (i.e., 100,000 µg/mL)
    - **Natamycin**: 10 mg / mL (i.e.10,000 µg/mL)
- Both preservatives were mixed with distilled water to prepare a stock solution.
- Once the bacterial culture in LB broth reached an **OD_{600} of 0.6**, indicating active logarithmic growth, it was considered ready for the MIC assay.

The culture was **divided into three separate sterile vials**:

- **Vial 1**: Containing Potassium **Sorbate**
- **Vial 2**: Containing **Natamycin**
- **Vial 3**: Served as the **untreated standard control** (no preservatives added)

And to them, 5 mL of the bacterial culture was added and kept in the shaker, and OD was checked at the interval of 1 hour.

The same process was repeated to obtain both individuals' and preservatives' minimum and maximum dosages.

Dosage- (Minimum range)

$C_1V_1 = C_2V_2$:

- C_1 = Concentration of the stock solution (µg/mL)
- V_1 = Volume of stock required (mL)
- C_2 = Desired concentration of working solution (µg/mL)
- V_2 = Final volume of working solution (mL)

For Potassium sorbate –

Minimum Concentration (0.01%) = 100 µg/mL

Desired final volume (V_2) = **5 mL**

Calculation for i.e., 0.01% (100 µg/mL):

C_1 = 100 mg/mL = 100,000 µg/mL (stock concentration)
C_2 = 100 µg/mL
V_2 = 5 mL

Using the formula:

$C_1V_1 = C_2V_2$

(100,000) (V_1) = (100) (5)

V_1 = 500 / 100,000

V_1 = 0.005 mL (or 5 µL)

For Natamycin –

Minimum Concentration = 50 µg/mL

Desired final volume (V_2) = **5 mL**

C_1 = 10 mg/mL = 10,000 µg/mL (stock concentration)
C_2 = 50 µg/mL
V_2 = 5 mL

$C_1V_1 = C_2V_2$

$10{,}000 \times V_1 = 50 \times 5$

$V_1 = 250 / 10{,}000$

V_1 = 0.025 mL = 25 µL

For *E. coli*

(TABLE 8) Starting OD – 0.65

Time	Standard	Potassium sorbate	Natamycin
1 pm	0.87	0.40	0.50
2 pm	0.94	0.33	0.43
3 pm	1.09	0.26	0.39

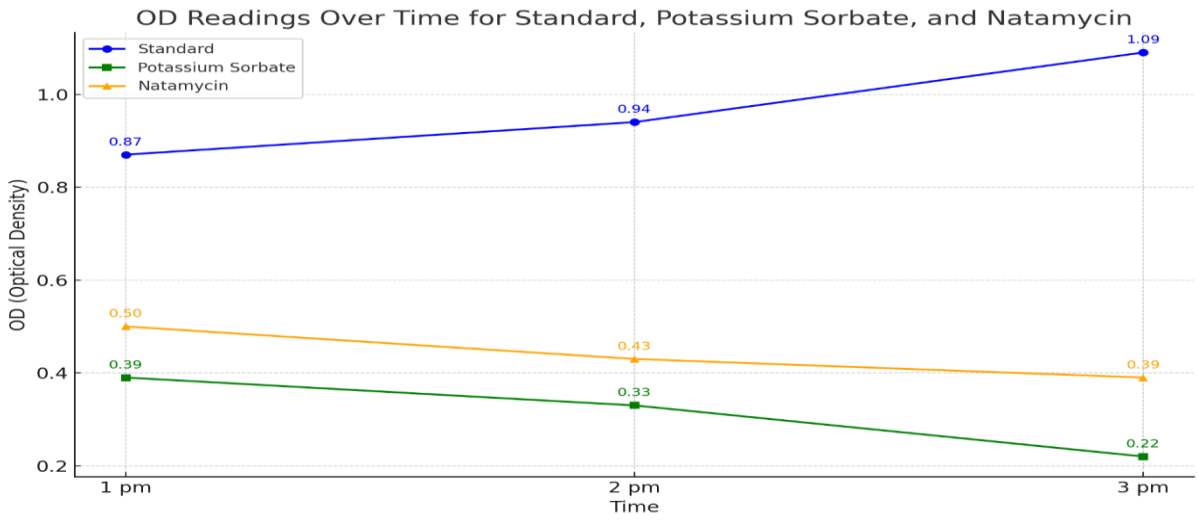


Fig.24(a): Minimum dosage of preservative in E. coli

(TABLE 9) For *S. aureus*

Starting OD = 0.65

Time	Standard	Potassium sorbate	Natamycin
1 pm	0.85	0.32	0.42
2 pm	0.92	0.25	0.37
3 pm	1.06	0.18	0.33

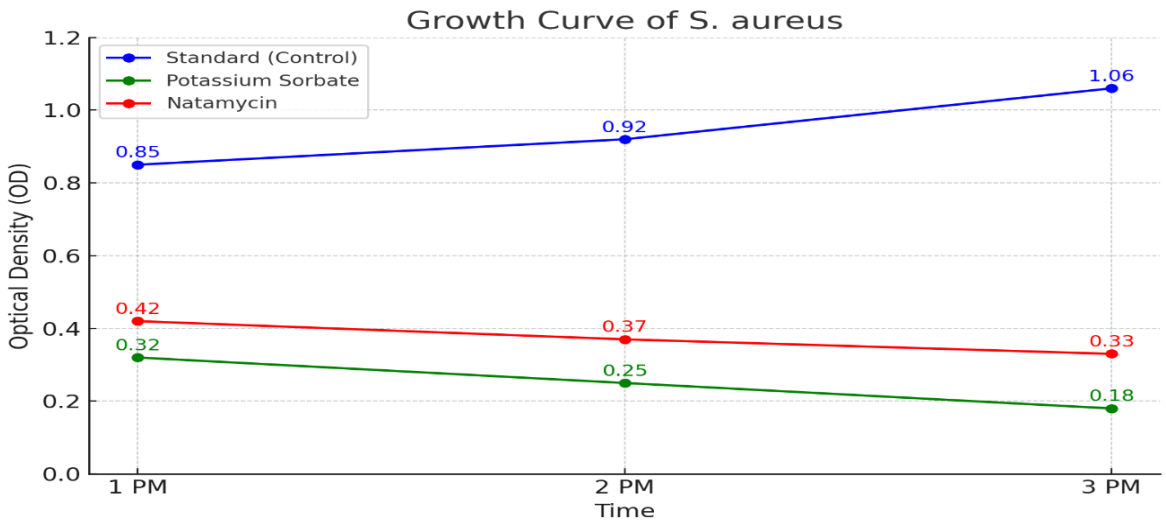


Fig. 24(b): Minimum range of preservatives in ***S. aureus***

Dosage (Maximum range):

For potassium sorbate

Maximum Concentration (0.05%) = 500 µg/mL

Desired final volume (V_2) = **5 mL**

Calculation for i.e., 0.05% (500 µg/mL):

C_1 = 100 mg/mL = 100,000 µg/mL (stock concentration)
C_2 = 500 µg/mL
V_2 = 5 mL

$C_1V_1 = C_2V_2$

(100,000) (V_1) = (500) (5) = $V_1 = 2500 / 100{,}000 =$ **V_1 = 0.025 mL (or 25 µL)**

For Natamycin -

Maximum Concentration = 50 µg/mL

Desired final volume (V_2) = **5 mL**

C_1 = 10 mg/mL = 10,000 µg/mL (stock concentration)
C_2 = 100 µg/mL
V_2 = 5 mL

$C_1V_1 = C_2V_2$

$10,000 \times V_1 = 100 \times 5$

$V_1 = 500 / 10,000$

V_1 = 0.05 mL = 50 µL

(TABLE 10) For *E. coli*

Starting OD – 0.63

Time	Standard	Potassium sorbate	Natamycin
4:30 pm	0.80	0.16	0.18
5:30 pm	0.86	0.14	0.08
6:30 pm	0.96	0.11	0.05

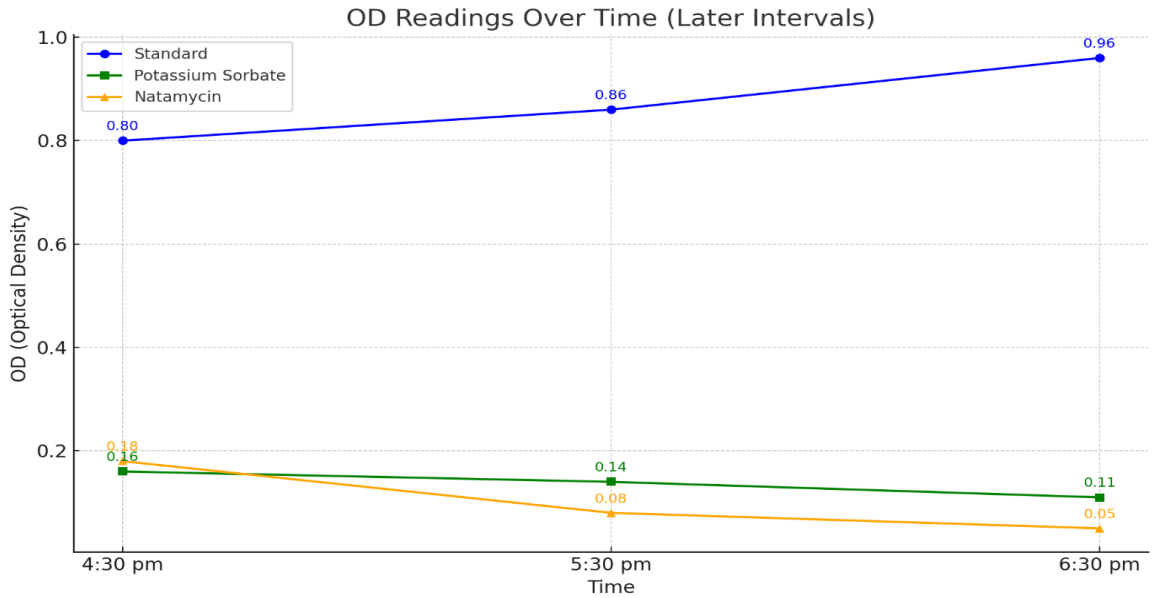


Fig.25(a): Maximum dosage of preservatives in *E. coli*

(TABLE 11) For *S. aureus*

Starting OD = 0.63

Time	Standard	Potassium sorbate	Natamycin
4:30 pm	0.78	0.12	0.10
5:30 pm	0.83	0.09	0.06
6:30 pm	0.92	0.06	0.03

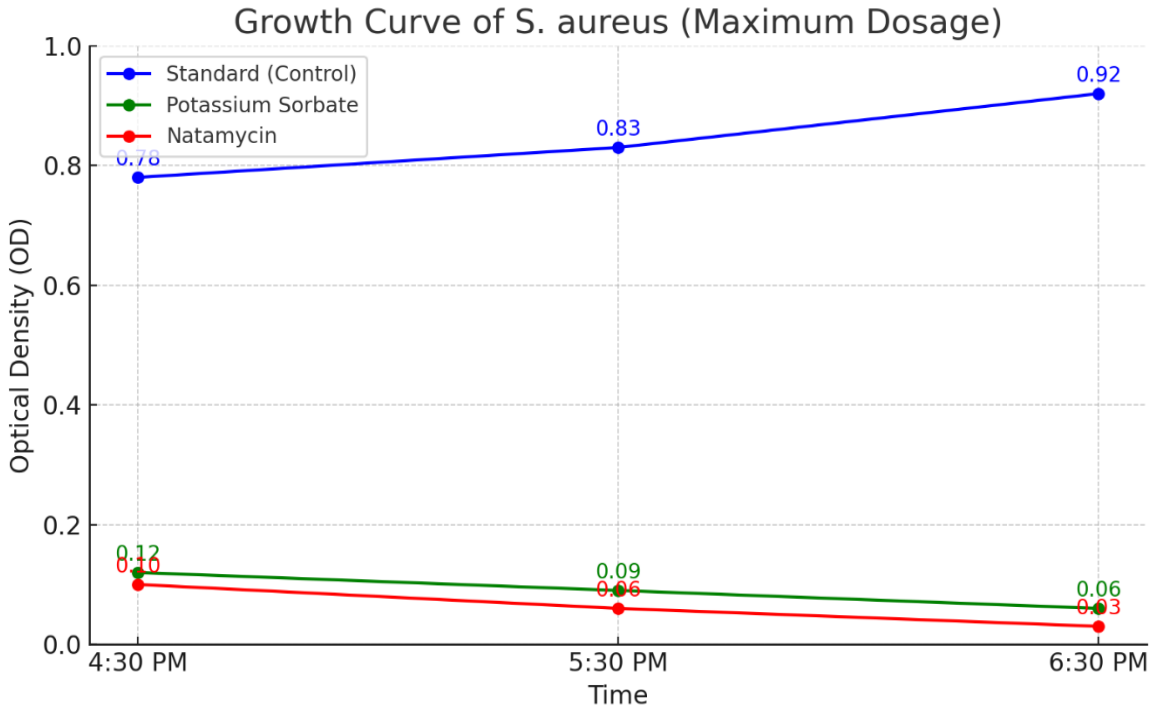


Fig.25(b): Maximum dosage of preservatives in ***S. aureus***

"After completing the individual Minimum Inhibitory Concentration (MIC) tests for each preservative, I performed the MIC analysis using a combination of the preservatives to assess their cumulative antimicrobial effect."

Here, also, the pure colonies of *E. coli* and *S. aureus* were inoculated in LB broth and kept in the shaker until 0.6 OD was obtained.

Now, for performing the mixed preservative test again, 3 vials were taken, one for the standard and the other two for the preservatives.

It is generally calculated in the 1:1 ratio

Dosage for the minimum range –

Potassium sorbate and Natamycin at minimum dose (100 mg/L and 50 mg/L)

Potassium Sorbate: 100 mg/L (0.1 mg/mL)

• Natamycin: 50 mg/L (0.05 mg/mL)

• Final Volume (V_2): 3 mL

• Stock Concentration (C_1): 1 mg/mL for both compounds

Potassium Sorbate: $V_1 = (0.1 \times 3) / 1 = 0.3$ mL

Natamycin: $V_1 = (0.05 \times 3) / 1 = 0.15$ mL

(TABLE 12) For *E. coli*

Starting OD – 0.62

Time	Standard	Minimum range
1 pm	0.66	0.42
2 pm	0.81	0.32
3 pm	0.92	0.11

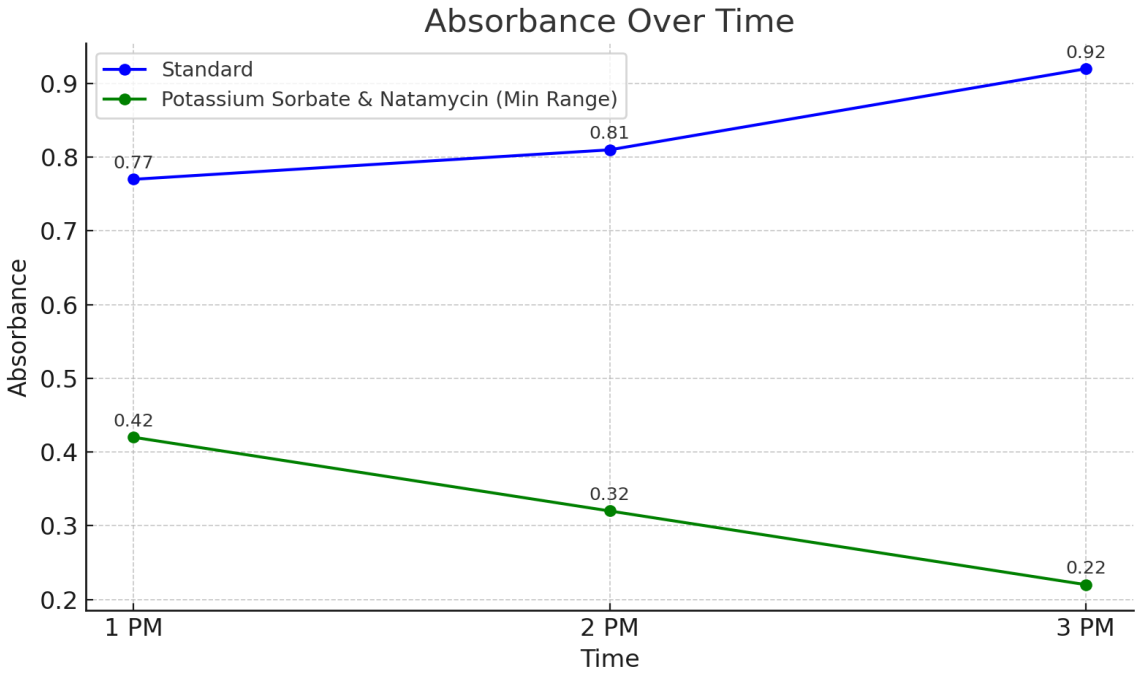


Fig.26(a): Minimum dosage of mixed preservatives on ***E. coli***

(TABLE 13) For *S. aureus*

Starting OD – 0.64

Time	Standard	Minimum range
1 pm	0.69	0.47
2 pm	0.87	0.37
3 pm	0.92	0.16

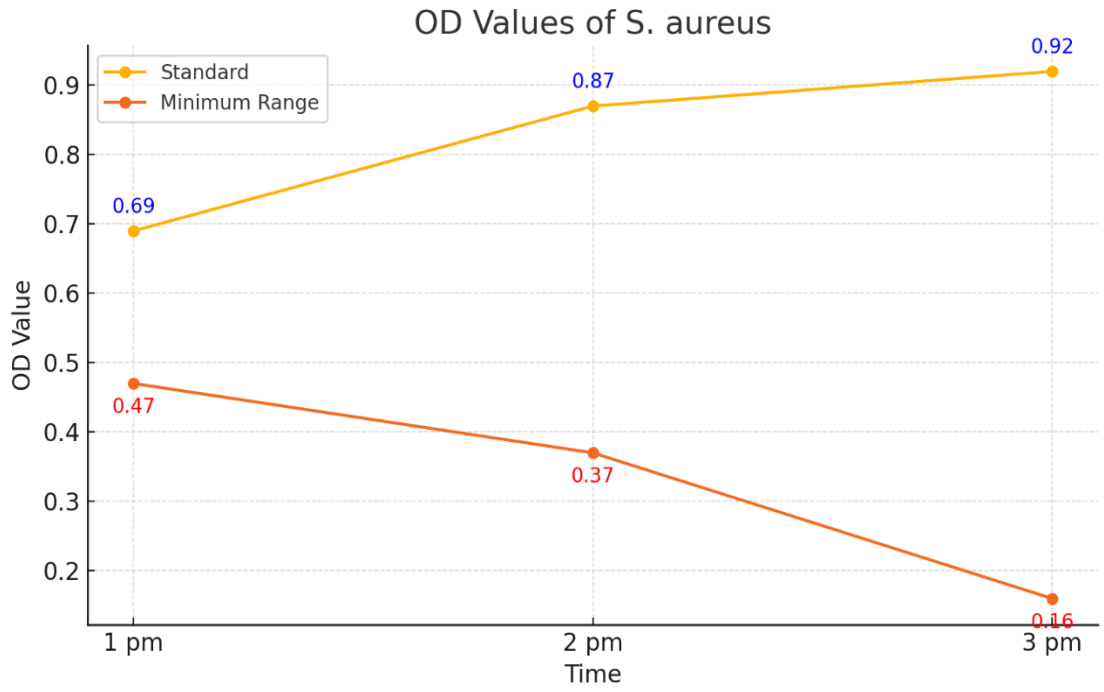


Fig.26(b): Minimum dosage of mixed preservatives on ***S. aureus***

Mixed preservative at maximum range –

Potassium Sorbate: 500 mg/L (0.5 mg/mL)

Natamycin: 100 mg/L (0.1 mg/mL)

• Final Volume (V_2): 3 mL

• Stock Concentration (C_1): 1 mg/mL for both compounds

Potassium Sorbate: V_1 = (0.5 × 3) / 1 = 1.5 mL

Natamycin: V_1 = (0.1 × 3) / 1 = 0.3 mL

(TABLE 14) For *E. coli*

Starting OD – 0.62

Time	Standard	Maximum range
10 am	0.66	0.16
11 am	0.79	0.06
12 pm	0.94	0.01

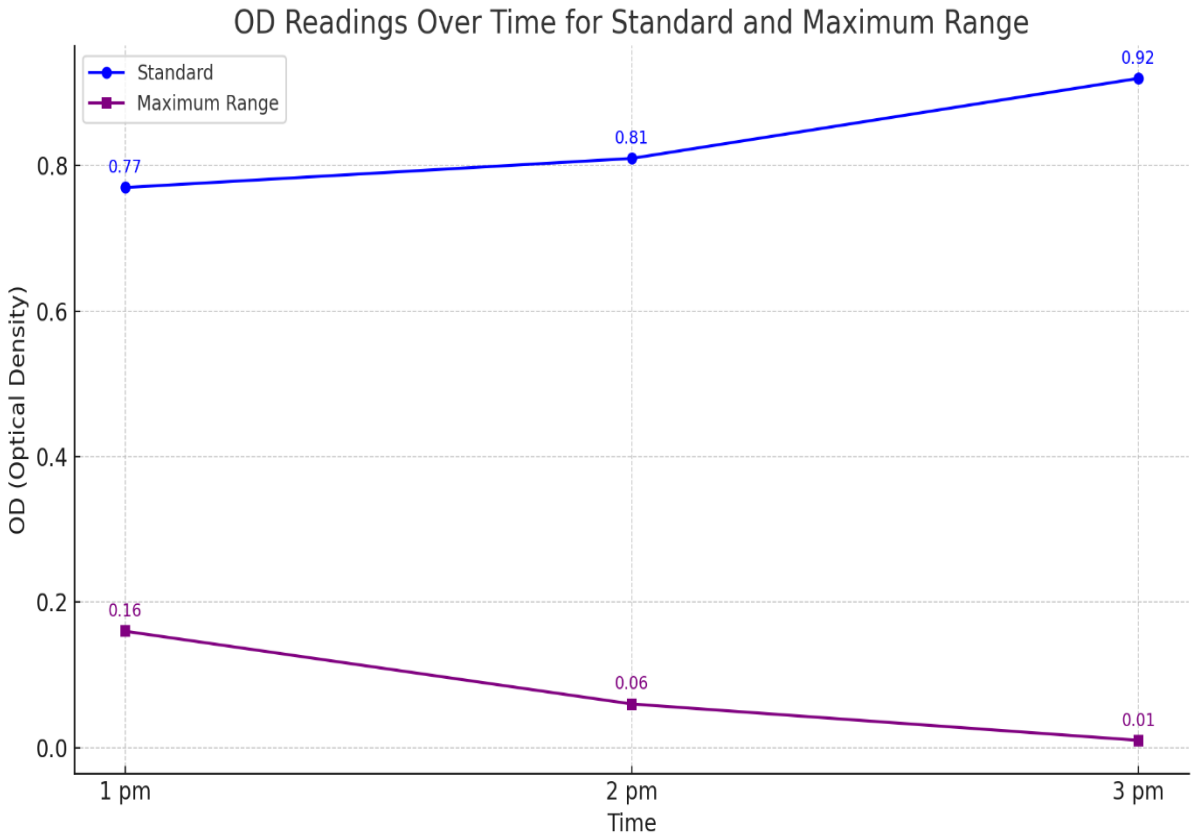


Fig.27(a): Maximum range of both preservatives on ***E. coli***

(TABLE 15) For *S. aureus*

Starting OD – 0.63

Time	Standard	Maximum range
10 am	0.66	0.11
11 am	0.79	0.01
12 pm	0.94	0.00

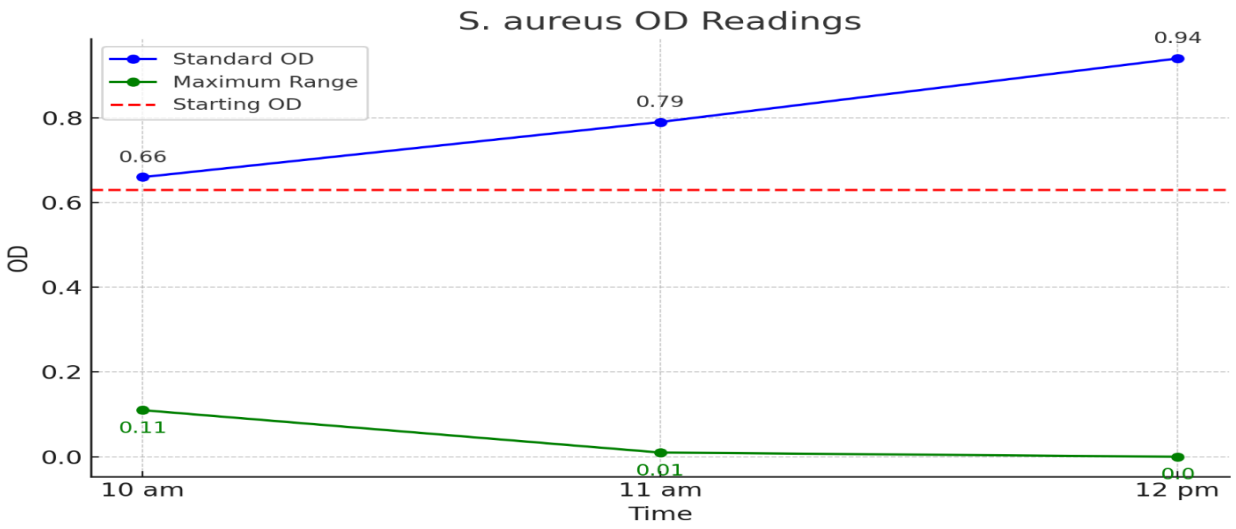


Fig.27(b): Maximum range of mixed preservatives on ***S. aureus***

RESULT AND DISCUSSION –

The present study focused on evaluating the antimicrobial activity of two commonly used artificial preservatives in milk—potassium sorbate and natamycin—against bacterial isolates from milk samples (*Escherichia coli* and *Staphylococcus aureus*). The antimicrobial resistance profiles were assessed using disk diffusion assays and minimum inhibitory concentration (MIC) testing, while the preservative levels were quantified using UV-visible spectrophotometry.

The results demonstrated a clear difference in the effectiveness of these preservatives. **Natamycin consistently exhibited strong antibacterial activity against *E. coli*, with a zone of inhibition measuring 18 mm (classified as sensitive) and MIC results confirming complete inhibition at higher concentrations.** Conversely, **potassium sorbate displayed only marginal activity against *E. coli* in the disk diffusion assay (10 mm, resistant), yet showed bacteriostatic effects at elevated concentrations in the MIC tests.** This highlights that potassium sorbate's antimicrobial activity is **concentration-dependent** and becomes more apparent when its MIC threshold is reached.

For ***S. aureus***, **natamycin's activity was intermediate (20 mm)** in the disk diffusion assay and only partially effective in MIC tests. **Potassium sorbate was also effective (12 mm),** reinforcing previous findings that ***S. aureus* is more resistant to common preservatives**. This can be attributed to mechanisms such as biofilm formation and the activity of efflux pumps, which reduce preservative penetration and increase bacterial tolerance.

The quantitative analysis of commercial milk samples revealed **average potassium sorbate and natamycin concentrations of approximately 48.88 mg/L and 39.04 mg/L, respectively.** These values align with permissible regulatory standards and confirm the presence of these preservatives in commercially processed milk. However, the observed antimicrobial resistance of ***S. aureus*** and partial resistance of ***E. coli*** suggest that **these preservatives, at commercial concentrations, may not fully inhibit bacterial growth in real-world milk storage conditions**.

Interestingly, the combined preservative tests (mixed preservative MIC tests) demonstrated **a modest enhancement in antibacterial activity against *E. coli*,** suggesting potential synergistic effects. Nevertheless, ***S. aureus* remained largely resistant even in mixed tests**, indicating that simply combining these preservatives may not overcome its adaptive resistance mechanisms. Overall, these findings highlight the **importance of testing preservatives not only for regulatory compliance (quantitative presence) but also for functional effectiveness against relevant pathogens**. They also reinforce the idea that preservatives like potassium sorbate may be less effective at standard usage concentrations, particularly against **Gram-positive bacteria like *S. aureus***.

CONCLUSION -

The findings of this study align with previously reported antimicrobial properties of natamycin and potassium sorbate. Natamycin, a polyene macrolide antifungal agent, has demonstrated bacteriostatic or bactericidal activity against certain bacteria, which explains its effectiveness against ***E. coli***. In contrast, potassium sorbate primarily acts as a fungistatic agent and shows limited bactericidal action, consistent with the observed resistance in both test organisms. The observed resistance of ***S. aureus*** to both preservatives highlights the complex challenge of controlling this pathogen in dairy environments. ***S. aureus*** is known for its adaptive resistance mechanisms, including biofilm formation and efflux pump activity, which likely contribute to the reduced susceptibility to preservatives in this study. The implications of these findings are significant. Although the preservatives tested are within regulatory limits and generally safe for milk preservation, their variable effectiveness against different bacterial species may contribute to selective pressure and the potential emergence of resistant strains. In particular, the observed sensitivity of ***E. coli*** to natamycin suggests its utility as an antimicrobial preservative in milk, but the partial or absent activity of potassium sorbate underscores the need for careful preservative selection or combination strategies to ensure microbial safety.

The emergence of AMR in foodborne pathogens is a critical concern. This study underscores the necessity of routine surveillance for AMR profiles in dairy settings and supports the need for alternative or complementary antimicrobial strategies, such as the use of bacteria or bacteriocins, to mitigate reliance on chemical preservatives.

Preservative effectiveness varies: Natamycin demonstrated superior antibacterial activity against ***E. coli*** compared to potassium sorbate. ***S. aureus*** poses a challenge: Both preservatives failed to achieve complete inhibition of S. aureus, suggesting the need for improved control measures in milk processing environments, except for maximum dosage, which is toxic to the human body. Regulatory implications: While preservative concentrations are within permissible limits, their limited antimicrobial effect against certain bacteria could necessitate re-evaluation of usage guidelines and combinations in milk preservation.

Future directions: Expanded studies including a broader range of milk samples, additional pathogens, and alternative preservatives or natural antimicrobials would provide deeper insights into the role of preservatives in mitigating AMR. In conclusion, this research provides a comprehensive evaluation of how milk preservatives influence antimicrobial resistance profiles in key dairy-related pathogens. It highlights both the promise and limitations of commonly used preservatives and calls for continued vigilance and innovation in food safety and quality control strategies.

APPENDIX –

Appendix A: Calibration Curves and Spectrophotometric Data

- Graph 1: Calibration curve of potassium sorbate at 254 nm
- Graph 2: Calibration curves of natamycin at 318.2 nm, 304 nm, and 296 nm

Absorbance Data and Concentration Calculations:
Potassium Sorbate (254 nm): Absorbance: 1.955, Concentration: 48.88 mg/L
Natamycin (318.2 nm): Absorbance: 0.593, Concentration: 23.10 mg/L
Natamycin (304 nm): Absorbance: 1.763, Concentration: 46.57 mg/L
Natamycin (296 nm): Absorbance: 1.828, Concentration: 49.45 mg/L
Average Natamycin Concentration: 39.04 mg/L

Appendix B: MIC Preparation and Calculation

Stock Solution Concentrations:
Potassium Sorbate: 100 mg/mL
Natamycin: 10 mg/mL

Minimum Range:
- Potassium Sorbate: 5 µL of stock in 5 mL (100 µg/mL)
- Natamycin: 25 µL of stock in 5 mL (50 µg/mL)

Total preservative volume: 30 µL, Diluent: 4.97 mL

Maximum Range:
- Potassium Sorbate: 25 µL of stock in 5 mL (500 µg/mL)
- Natamycin: 50 µL of stock in 5 mL (100 µg/mL)

Total preservative volume: 75 µL, Diluent: 4.925 mL

Appendix C: AMR Test Data

Zone of Inhibition Data:

S. aureus:
- Potassium Sorbate: 12 mm (Resistant)
- Natamycin: 15 mm (Intermediate)
- Control: 0 mm (Resistant)

E. coli:
- Potassium Sorbate: 10 mm (Resistant)
- Natamycin: 18 mm (Sensitive)
- Control: 0 mm (Resistant)

MIC OBSERVATION –

Potassium sorbate was effective at higher concentrations in broth MIC tests, indicating concentration-dependent bacteriostatic activity.

Natamycin showed consistent activity against E. coli, while S. aureus exhibited partial inhibition.

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– Explores links between environmental stress (like preservatives) and resistance, a valuable context for your AMR focus.
19. **Klaenhammer, T.R. (1988). Bacteriocins of Lactic Acid Bacteria. Biochimie, 70(3), 337-349.**
– Provides context on natural antimicrobials from lactic acid bacteria, relevant for discussing competitive or synergistic preservation.
20. **Gänzle, M.G. (2015). Lactic Metabolism Revisited: Metabolism of Lactic Acid Bacteria in Food Fermentations and Food Spoilage. Current Opinion in Food Science, 2, 106-117.**
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Abstract

Milk is a staple dietary component rich in essential nutrients, but it is often subjected to adulteration and preservation using chemical agents. Both commercial and shed (locally sourced) milk samples were analysed for the presence of these compounds using spectrophotometric techniques. Calibration curves for potassium sorbate and natamycin were plotted. Their absorbance was measured at specific wavelengths (254 nm for potassium sorbate and 318.2, 304, 296 nm for natamycin) and applied to determine preservative concentrations in unknown samples.

Microbial isolates such as E. coli and Staphylococcus aureus were obtained from the milk samples and tested for antimicrobial susceptibility via the MIC (Minimum Inhibitory Concentration) method. Nutrient agar was used for microbial growth, followed by serial dilution of milk and antimicrobial assays. Biochemical tests were employed to confirm microbial identity, including urease, catalase, and sugar fermentation.

The results revealed potassium sorbate concentration in milk at approximately 48.88 mg/L and natamycin at an average of 39.04 mg/L. These concentrations demonstrated varying effects on microbial growth, indicating resistance in some isolates. The data suggest a potential link between preservative exposure and emerging antimicrobial resistance in foodborne microbes. This raises concerns about long-term public health impacts and regulatory policies concerning food preservatives. The project highlights the importance of routine monitoring of milk quality and calls for stricter guidelines on the use of preservatives in food industries to mitigate the risk of AMR development.

Keywords –

Antimicrobial resistance (AMR), milk preservatives, potassium sorbate, natamycin, Escherichia coli, Staphylococcus aureus, minimum inhibitory concentration (MIC), food adulterants, spectrophotometric analysis, bacterial isolates, commercial milk, shed milk, food safety, UV-Visible spectrophotometry, microbial tolerance

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scientific literature but also offer actionable insights for improving food quality assurance, public health policies, and consumer awareness in India and beyond.

This study aims to investigate the influence of selected artificial preservatives and adulterants on antimicrobial resistance patterns in bacterial isolates obtained from raw (shed) and commercially available milk. It also integrates both wet lab techniques, such as Minimum Inhibitory Concentration (MIC) testing and biochemical profiling, with computational approaches like molecular docking and predictive modeling. Through this multidisciplinary approach, the study seeks to generate robust data that can inform food safety regulations and raise awareness about the silent but critical threat posed by AMR arising from food sources.

Plagiarized Sources

In India, the Food Safety and Standards Authority of India (FSSAI) serves as the apex body for ensuring the safety and hygiene of food products, including milk and dairy items.

https://edukemy.com/blog/fssai-upsc

This study aims to investigate the influence of selected artificial preservatives and adulterants on antimicrobial resistance patterns in bacterial isolates obtained from raw (shed) and commercially available milk.

https://pmc.ncbi.nlm.nih.gov/articles/PMC7198526

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LITERATURE REVIEW –

Milk, a nutritionally rich fluid, is widely consumed across the globe. Its high nutrient content, while beneficial for human health, also makes it a suitable medium for microbial growth. Consequently, milk is often treated with artificial preservatives to ensure its shelf life and microbial safety. However, the long-term use and consumption of such preservatives raise concerns regarding antimicrobial resistance (AMR) and potential health hazards.

Several studies have documented the frequent use of preservatives such as potassium sorbate and natamycin in milk and dairy products to inhibit yeast, mold, and certain bacteria (Gautam et al., 2021). While regulatory agencies generally recognize these compounds as safe (GRAS) when used within permissible limits, emerging evidence suggests that prolonged exposure may induce bacterial adaptation and resistance (El-Sayed et al., 2020).

Antimicrobial resistance in foodborne bacteria is a global concern, with the World Health Organization recognizing AMR as a major threat to food safety. A study by Bhattacharjee et al. (2019) highlighted the resistance patterns of E. coli and Staphylococcus aureus isolated from milk samples, indicating that even non-therapeutic exposure to preservatives might select for resistant strains over time.

The adulteration of milk with chemical agents, either to mask spoilage or to alter composition, further complicates the problem. Substances like detergent, urea, and formalin are sometimes illegally added, and their residues can influence microbial behavior and resistance profiles (Kumar & Sharma, 2018). These additives not only pose toxicological risks but may also interfere with the antimicrobial susceptibility of bacteria by altering pH or damaging cellular structures.

Research using Minimum Inhibitory Concentration (MIC) assays and molecular docking studies has shown that preservatives such as potassium sorbate may interact with bacterial enzymes, contributing to adaptive resistance mechanisms (Singh et al., 2022). Computational tools also allow the prediction of resistance gene activation, offering insights into how sublethal preservative exposure may lead to AMR.

Emerging alternatives like plant-based antimicrobials and probiotics are being explored to mitigate the issue, but artificial preservatives remain widely used due to their cost-effectiveness and ease of application. Therefore, continuous surveillance and a deeper understanding of their role in AMR are crucial, especially in countries where milk preservation methods may lack stringent regulatory oversight.

Recent advances in in vitro and in silico methodologies have shed light on the mechanisms of preservative-induced resistance. Minimum Inhibitory Concentration (MIC) assays are widely used to quantify bacterial resistance levels, while molecular docking studies suggest that compounds like potassium sorbate may bind to key microbial enzymes, thereby disrupting metabolic pathways and prompting compensatory resistance responses (Singh et al., 2022). Computational tools such as predictive modeling and resistance gene mapping are now being utilized to identify how preservative

exposure may trigger efflux pumps or gene expression related to AMR.
Alternative strategies, such as plant-derived antimicrobial compounds, bacteriocins, and probiotics, are currently being researched as more sustainable approaches to milk preservation. Nonetheless, artificial preservatives continue to dominate due to their lower cost, extended shelf stability, and ease of industrial integration. As a result, the balance between food safety, microbial control, and public health risk remains precarious.
Given these complexities, there is a pressing need for continuous surveillance of preservative usage and deeper investigation into its microbial consequences. Countries like India, with rapidly expanding dairy industries, must especially uphold and enforce FSSAI guidelines alongside international frameworks such as Codex Alimentarius and HACCP (Hazard Analysis and Critical Control Points) to monitor preservative usage and assess its impact on bacterial ecology.
This project seeks to bridge this knowledge gap by assessing AMR patterns in bacterial isolates from milk treated with common preservatives. By correlating preservative concentrations with bacterial resistance via laboratory and computational approaches, the study aims to comprehensively evaluate the microbiological and public health risks associated with these additives.

Plagiarized Sources

Antimicrobial resistance in foodborne bacteria is a global concern, with the World Health Organization recognizing AMR as a major threat to food safety.
https://www.fao.org/fao-who-codexalimentarius/thematic-areas/antimicrobial-resistance/en

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1. METHODS AND MATERIALS

• Isolation of microorganisms- Firstly, shed milk was collected from the shed. After that, 20 ml of nutrient broth was prepared and. autoclaved the medium at 121°C and 15 psi pressure for 15 minutes to sterilize it completely.

INGREDIENTS AMOUNT in gm

Peptone 0.2g

Meat extract 0.2g

Sodium chloride 0.1g

Agar 0.45g

Serial Dilution of Shed Milk Sample (Up to 10^3 Dilution):

• Label three sterile test tubes or microcentrifuge tubes as 10^1, 10^2, and 10^3.

• Add 900 µL of sterile 0.9% NaCl solution (normal saline) into each of the three tubes.

• Using a sterile micropipette or pipette:

o Take 100 µL of the shed milk sample and add it to the first tube (10^1) containing 900 µL of NaCl.

o Mix thoroughly by pipetting up and down or by vortexing gently.

• From the 10^1 dilution tube:

o Take 100 µL and add it into the second tube (10^2) containing 900 µL NaCl.

o Mix thoroughly again.

• From the 10^2 dilution tube:

o Take 100 µL and add it into the third tube (10^3) containing 900 µL NaCl.

o Mix thoroughly.

• Now, you have 10^1, 10^2, and 10^3 serial dilutions ready for plating.

Plating the Diluted Samples:

• Using a sterile pipette tip, take 100 µL from the 10^3 dilution tube.

• Spread the 100 µL sample evenly on a solidified NA plate's surface using a sterile L-shaped or glass spreader.

• Allow the sample to absorb into the agar.

• Incubate the plates inverted at 37°C for 24 hours (or until visible colonies). Fig.1: Growth of microorganisms

Pure colony Isolation

• Using a sterile inoculating loop, pick individual colonies and streak them onto fresh nutrient agar plates to obtain pure cultures.

• Incubate the streaked plates at 37°C for another 24 hours.

• Biochemical tests were performed on pure colonies to identify bacterial species.

BIOCHEMICAL TESTS-

Catalase Test

• A loopful of bacterial colony was placed on a clean slide.

• A drop of 3% hydrogen peroxide was added.

• Immediate bubble formation indicated a positive catalase reaction.

Indole Test-

• The bacteria were inoculated into tryptone broth and incubated at 37°C for 24-48 hours.

• After incubation, 5 drops of Kovac's reagent were added carefully along the sides.

• Pink colour rings form.

Simmon Citrate Test:

• The pure colony was streaked into an agar slant.

• After 24-48 hours of incubation, a color change from green to blue indicated positive citrate utilization.

Methyl Red Test (MR)

• The pure colony was incubated in MR-VP broth and incubated at 37°C for 48 hours.

• Add a few drops of Methyl Red indicator after incubation.

• Observe the color change to red

Voges-Proskauer Test (VP)

• After incubation in MR-VP broth, add:

o 0.6 mL of alpha-naphthol.

o 0.2 mL of KOH solution.

• Mix gently and leave for 15–30 minutes.

• Observe color change.

Glucose Fermentation Test

• Inoculate bacteria into glucose fermentation broth containing phenol red and a Durham tube.

• Incubate at 37°C for 24-48 hours.

• Check for:

o Colour change (acid production).

o Gas bubble formation (gas production).

Urease Test-

• The colony is inoculated onto the surface of a urea agar slant.

• And then it was incubated at 37°C for 24–48 hours.

• Observe for colour change.

Gram staining-

1. A clean glass slide was taken, and a thin smear of bacterial culture from the milk sample was prepared using a sterile inoculating loop.

2. The smear was air-dried and then heat-fixed by gently passing it over a flame.

3. The slide was flooded with crystal violet stain for one minute and then gently rinsed with distilled water.

4. Gram's iodine solution was added and left for one minute to act as a mordant, followed by gentle rinsing.

5. The slide was then decolorized with 95% ethanol for 15 20 seconds and immediately rinsed with water to prevent over-decolorization.

6. Safranin counterstain was applied for one minute, and the slide was rinsed and air-dried.

7. The stained slide was observed under a light microscope using a 100x oil immersion objective.

Observation:

• Purple-coloured cells indicated Gram-positive bacteria.

• Pink/red-coloured cells indicated Gram-negative bacteria.

Based on Gram staining results:

• Isolates showing purple colour were identified as Gram-positive bacteria (e.g., Staphylococcus aureus).

• Isolates showing pink colour were identified as Gram-negative bacteria (e.g., Escherichia coli).

Plagiarized Sources

• **Add a few drops of Methyl Red indicator after incubation.**

https://chemidp-test.acs.org/methyl-red-voges-proskauer-test

• **The colony is inoculated onto the surface of a urea agar slant.**
https://microbenotes.com/urease-test-principle-procedure-and-result

3. The slide was flooded with crystal violet stain for one minute and then gently rinsed with distilled water.
https://www.cliffsnotes.com/study-notes/23784767

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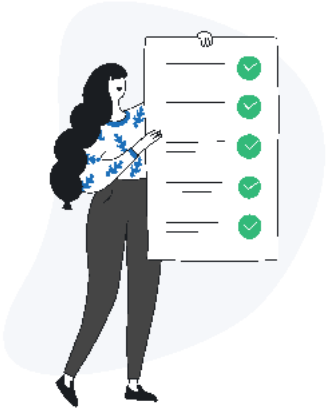
Sugar Test –
Three Eppendorf tubes were taken:
• One tube was used as a standard control (glucose solution).
• Two tubes were used for the unknown samples (shed milk and commercial milk). Into the standard control tube, 500 µL of glucose solution was added. To the standard, 0.1 mL of Fehling's A and 0.1 mL of Fehling's B solutions were added slowly and mixed. Similarly, 500 µL of shed milk and 500 µL of commercial milk were taken into the other two tubes, respectively, and the amount was slowly increased.

i. Before adding anything, the empty weight of each Eppendorf tube was measured and recorded.
ii. To each sample, 0.1 mL of Fehling's A and 0.1 mL of Fehling's B solutions were added slowly.

iii. Methylene blue was added dropwise to each Eppendorf tube containing the samples until a visible colour change started, from blue to the beginning of red precipitate formation.
iv. The tubes were allowed to rest undisturbed for 10 20 minutes to ensure complete precipitation of the brick-red cuprous oxide.
v. After completion, the amount of sugar present was calculated based on the precipitate formation and compared with the standard control.



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Total Words: 147 Total Characters: 817 Plagiarized Sentences: 0 Unique Sentences: 9 (100%)

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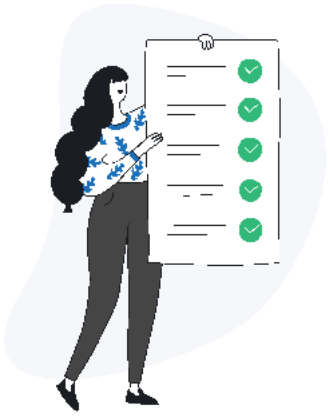
1. Standard and Sample Preparation
- Take 0.2 to 1 mL of each BSA standard in separate test tubes.
- Add 0.2 mL of shed milk and commercial milk samples into two additional tubes.
- Make the volume in each tube up to 1 mL with distilled water.

2. Addition of Reagents
- 5 mL of Lowry's reagent (Reagent A) was added to all tubes (standards and sample.
- After that, there were 10 minutes of incubation.
- 0.5 mL of diluted Folin–Ciocalteu reagent was given to each test tubes and mixed immediately.

3. Incubation and Color Development
- Incubate all tubes in a dark place or water bath at room temperature (25–30°C) for 30 minutes to allow blue color development.

4. Measurement
- Measure the absorbance at 660 nm in a spectrophotometer.
- Use a blank (with all reagents but no protein) to calibrate the instrument.



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Total Words: 186 **Total Characters:** 1090 **Plagiarized Sentences:** 0.98 **Unique Sentences:** 13.02 (93%)

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FAT ESTIMATION –
i. 20 mL each of shed milk and commercial milk were taken separately in two beakers. (shed milk was 7.1 and commercial milk was 6.51)

ii. A 50:50 dilution of distilled water and 1% HCl solution was prepared. The diluted acid was slowly added to the milk samples while pH monitoring.

iii. Acid was added until the pH dropped to around 4 or lower, indicating the beginning of milk coagulation.

iv. After approximately five minutes, coagulation started visibly. The milk samples were then heated to 50–60°C to enhance coagulation.

After sufficient coagulation, the milk samples were filtered to separate the solid curd from the filtrate. The filtrate was collected carefully for fat estimation.

vi. Empty Petri plates were weighed before use.
 Shed milk Petri plate weight before = 47.56 g
 Commercial milk Petri plate weight before = 43.74 g
Fat Extraction using Petroleum Ether:
o For shed milk:
 15 mL of petroleum ether was added to the filtrate.
o For commercial milk:
 10 mL of petroleum ether was added.
o The samples were allowed to extract the fat content.

Plagiarized Sources

10 mL of petroleum ether was added.
https://www.chegg.com/homework-help/questions-and-answers/continued-picture-6-ml-isopropyl-alcohol-obtained-placed-beaker-hot-isopropyl-alcohol-adde-q89755525

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Total Words: 240 **Total Characters:** 1499 **Plagiarized Sentences:** 0 **Unique Sentences:** 15 (100%)

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PRESERVATIVE ESTIMATION IN COMMERCIAL MILK
i. A certain amount of milk was taken and heated to 60 degrees for 15 minutes.
i. HCL was added gradually so that the whole lump of casein was formed.
ii. After that, the whole milk was filtered off.
iii. To the filtrate, sodium bicarbonate was added to neutralize the acetic acid by checking the pH up to 7, and then the whole solution was boiled for at least 15 minutes.
iv. A whole bunch of precipitate was formed at the bottom and filtered off.
v. The filtrate was taken, then subsequently assayed for potassium sorbate and natamycin.
vi. Potassium sorbate and natamycin peaks were taken in the blank as distilled water.
vii. Potassium sorbate absorbance wavelength was 254nm, and natamycin was at – 318.2,304 and 296nm.
Standard Calibration Curve Preparation and Concentration Determination -
Potassium sorbate -
• 10, 20, 30, and 40 mg/L of standard solutions were prepared for potassium sorbate.
• A 254 nm UV-Vis spectrophotometer was used. and the absorbance values were recorded. A calibration graph was plotted with concentration (mg/L) on the X-axis and absorbance on the Y-axis
Natamycin –
• Standard solutions of natamycin were prepared in distilled water at known concentrations, like the potassium sorbate standards.
• Absorbance readings were recorded at three characteristic wavelengths of natamycin: 318.2 nm, 304 nm, and 296 nm.
• Separate calibration curves were plotted for each wavelength using their corresponding absorbance data.

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Total Words: 253 **Total Characters:** 1527 **Plagiarized Sentences:** 1.05 **Unique Sentences:** 13.95 (93%)

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Antimicrobial Resistance Test
Preparation of Solutions:
1. Preparation of Nutrient Agar:
• Dissolve the recommended amount of nutrient agar powder in distilled water (as per manufacturer's instructions).
• Boil until fully dissolved.
• Autoclaving at 121°C for 15 minutes for sterilization.
• Pour into sterile petri plates and allow to solidify at room temperature.
2. Preparation of Potassium Sorbate and Natamycin Stock Solutions:
• Weigh 1 g of potassium sorbate and 1 g of natamycin separately.
• Dissolve each in 10 mL of distilled water to make 100 mg/mL stock solutions.
• Prepare fresh and use immediately for best results.
3. Control Solution:
• Sterile distilled water (50 µL) as the negative control.

Procedure:
1. Inoculation of Bacteria:
• Prepare fresh bacterial cultures (E. coli and S. aureus).
• Spread 100 µL of bacterial suspension evenly on nutrient agar plates using a sterile spreader.
• Let the plates dry for 5 minutes at room temperature.
2. Well Diffusion Method:
• A sterile cork borer was used to punch three wells into the agar in each plate.
• After that, the agar plugs were placed without damaging the base.
3. Application of Test Solutions:
• To each well, 50 µL of each preservative was added:
o Well 1: Potassium Sorbate
o Well 2: Natamycin
o Well 3: Sterile distilled water (Control)
4. Incubation:
• Incubated at 37°C for 24 hours.
5. Observation:
• After incubation, the zones of inhibition were observed.
• Measure the diameter of inhibition zones in millimetres (mm) using a scale.

Plagiarized Sources

• **Autoclaving at 121°C for 15 minutes for sterilization.**

https://www.researchgate.net/profile/Shahira-El-Moslamy/publication/326271499_Statistical_Bioprocess_Strategies_for_Bio-Fabrication_of_Nano-Ag_from_Streptomyces_rectiviolaceus_Strain_SMWN32_as_a_Novel_Antimicrobial_Agent_against_Hospital-Acquired_Infectious_Pathogens/links/5ffd6db645851553a03a21be/Statistical-Bioprocess-Strategies-for-Bio-Fabrication-of-Nano-Ag-from-Streptomyces-rectiviolaceus-Strain-SMWN32-as-a-Novel-Antimicrobial-Agent-against-Hospital-Acquired-Infectious-Pathogens.pdf

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Total Words: 346 **Total Characters:** 2158 **Plagiarized Sentences:** 1.08 **Unique Sentences:** 16.92 (94%)

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Assessment of MIC for Potassium Sorbate and Natamycin
Preparation of Inoculum-15 mL of sterile Luria Bertani (LB) broth was prepared in a clean, sterile conical tube.
i. Using a sterile inoculation loop, bacterial colonies (previously isolated on nutrient agar plates) were picked and transferred into the LB broth.
ii. The inoculated broth was placed in a shaker and incubator at 37 °C and agitated at 150 rpm.
iii. The incubation continued until the bacterial culture's optical density (OD) reached 0.6, indicating the log phase of bacterial growth. This culture was then used as the standardized inoculum for testing.
Preparation of Preservative Solutions
• Potassium Sorbate and Natamycin were selected as the preservatives to be tested for their antimicrobial activity.
• Based on their solubility:
o Potassium Sorbate: 100 mg/mL (i.e., 100,000 µg/mL)
o Natamycin: 10 mg / mL (i.e.10,000 µg/mL)
• Both preservatives were mixed with distilled water to prepare a stock solution.
• Once the bacterial culture in LB broth reached an OD of 0.6, indicating active logarithmic growth, it was considered ready for the MIC assay.
The culture was divided into three separate sterile vials:
• Vial 1: Containing Potassium Sorbate
• Vial 2: Containing Natamycin
• Vial 3: Served as the untreated standard control (no preservatives added)
And to them, 5 mL of the bacterial culture was added and kept in the shaker, and OD was checked to the interval of 1 hour.
The same process was repeated to obtain both individuals' and preservatives' minimum and maximum dosages.

Dosage- (Minimum range)
C V = C V is followed

"After completing the individual Minimum Inhibitory Concentration (MIC) tests for each preservative, I performed the MIC analysis using a combination of the preservatives to assess their cumulative antimicrobial effect."
Here, also, the pure colonies of E. coli and S. aureus were inoculated in LB broth and kept in the shaker until 0.6 OD was obtained.

Now, for performing the mixed preservative test again, 3 vials were taken, one for the standard and the other two for the preservatives.
It is generally calculated in the 1:1 ratio

Plagiarized Sources

ii. The inoculated broth was placed in a shaker and incubator at 37 °C and agitated at 150 rpm.
https://pmc.ncbi.nlm.nih.gov/articles/PMC4477212

The culture was divided into three separate sterile vials:
https://pmc.ncbi.nlm.nih.gov/articles/PMC105314

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Total Words: 345 **Total Characters:** 2604 **Plagiarized Sentences:** 0 **Unique Sentences:** 17 (100%)

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RESULT AND DISCUSSION –

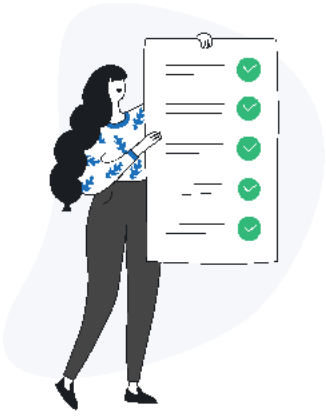
The present study was designed to evaluate the antimicrobial effectiveness of two commonly used milk preservatives—potassium sorbate and natamycin—against bacterial isolates (Escherichia coli and Staphylococcus aureus) and to assess their potential contribution to antimicrobial resistance (AMR). Additionally, the presence of these preservatives in milk samples was quantified using spectrophotometric analysis and standard calibration curves. Initially, standard solutions of potassium sorbate and natamycin were prepared in a range of concentrations, and absorbance values were measured to create calibration curves. These calibration curves facilitated accurate quantification of preservative levels in milk samples. The analysis revealed concentrations of potassium sorbate at approximately 48.88 mg/L and natamycin at an average of 39.04 mg/L in the commercial milk samples tested. These levels were within the regulatory standards for milk preservatives, indicating compliance with permissible limits.

The study then proceeded to evaluate the antimicrobial resistance of E. coli and S. aureus isolated from shed milk and commercial milk samples. Isolates were subjected to biochemical identification tests, confirming the presence of these indicator organisms. AMR testing was conducted using the disk diffusion method on nutrient agar plates, incorporating the preservatives individually and in combination. Zones of inhibition were carefully measured to determine sensitivity, intermediate response, or resistance.

The Minimum Inhibitory Concentration (MIC) tests conducted in this study demonstrated that both potassium sorbate and natamycin exhibit antimicrobial activity against Escherichia coli and Staphylococcus aureus. The MIC values indicated that natamycin generally requires a lower concentration to inhibit bacterial growth compared to potassium sorbate, especially against S. aureus, suggesting higher potency.

E. coli was found to be sensitive to both preservatives, with potassium sorbate showing slightly better efficacy at lower concentrations. In contrast, S. aureus showed greater susceptibility to natamycin, highlighting its potential as an effective antimicrobial agent against Gram-positive bacteria. These findings confirm that both potassium sorbate and natamycin can inhibit the growth of common milk contaminants, although their effectiveness varies with bacterial species and concentration. The MIC results provide essential data for determining optimal preservative levels to ensure microbial safety and extend the shelf life of milk products.

They also reinforce the idea that preservatives like potassium sorbate may be less effective at standard usage concentrations, particularly against Gram-positive bacteria like S. aureus.



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CONCLUSION -

The findings of this study align with previously reported antimicrobial properties of natamycin and potassium sorbate. Natamycin, a polyene macrolide antifungal agent, has demonstrated bacteriostatic or bactericidal activity against certain bacteria, which explains its effectiveness against E. coli. In contrast, potassium sorbate primarily acts as a fungistatic agent and shows limited bactericidal action, consistent with the observed resistance in both test organisms.

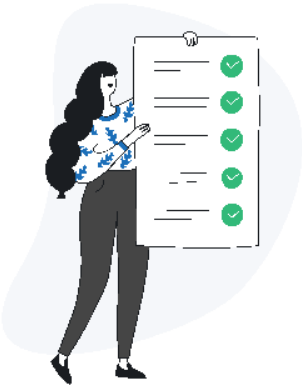
The observed resistance of S. aureus to both preservatives highlights the complex challenge of controlling this pathogen in dairy environments. S. aureus is known for its adaptive resistance mechanisms, including biofilm formation and efflux pump activity, which likely contribute to the reduced susceptibility to preservatives in this study. The implications of these findings are significant. Although the preservatives tested are within regulatory limits and generally safe for milk preservation, their variable effectiveness against different bacterial species may contribute to selective pressure and the potential emergence of resistant strains. In particular, the observed sensitivity of E. coli to natamycin suggests its utility as an antimicrobial preservative in milk, but the partial or absent activity of potassium sorbate underscores the need for careful preservative selection or combination strategies to ensure microbial safety.

The emergence of AMR in foodborne pathogens is a critical concern. This study underscores the necessity of routine surveillance for AMR profiles in dairy settings and supports the need for alternative or complementary antimicrobial strategies, such as the use of bacteria or bacteriocins, to mitigate reliance on chemical preservatives.

Preservative effectiveness varies: Natamycin demonstrated superior antibacterial activity against E. coli compared to potassium sorbate. S. aureus poses a challenge: Both preservatives failed to achieve complete inhibition of S. aureus, suggesting the need for improved control measures in milk processing environments, except for maximum dosage, which is toxic to the human body.

Regulatory implications: While preservative concentrations are within permissible limits, their limited antimicrobial effect against certain bacteria could necessitate re-evaluation of usage guidelines and combinations in milk preservation.

Future directions: Expanded studies including a broader range of milk samples, additional pathogens, and alternative preservatives or natural antimicrobials would provide deeper insights into the role of preservatives in mitigating AMR. In conclusion, this research provides a comprehensive evaluation of how milk preservatives influence antimicrobial resistance profiles in key dairy-related pathogens. It highlights both the promise and limitations of commonly used preservatives and calls for continued vigilance and innovation in food safety and quality control strategies.



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